VOLUME 39 JUNE 1961 NUMBER 3

Canadian Journal of Zoology

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Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

Canadian Journal of Zoology

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Canadian Journal of Zoology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

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SPOROGONY OF LEUCOCYTOZOON AND HAEMOPROTEUS IN SIMULIIDS AND CERATOPOGONIDS AND A REVISED CLASSIFICATION OF THE HAEMOSPORIDIIDA[1]

A. M. FALLIS AND G. F. BENNETT

Abstract

Sporogony of species of *Leucocytozoon* and *Haemoproteus* in simuliids and ceratopogonids respectively is described and illustrated. Oöcysts of both types of parasites are only slightly larger than the zygotes and contain relatively few sporozoites at maturity. Sporogony of some individuals is completed in fewer than 5 days at approximately 22° C; others may require 14–18 days. Comparisons of the morphology and life histories of these parasites with those of *Plasmodium* suggest that the three genera should be placed in separate families.

Introduction

Ornithophilic simuliids and ceratopogonids in Algonquin Park, Canada, are known hosts of certain species of *Leucocytozoon* and *Haemoproteus* respectively (10–14). Unfortunately, the larval stages of some of these flies have yet to be located in Algonquin Park. This, together with the difficulties of rearing these to obtain parasite-free insects and having them feed in the laboratory, has delayed investigations of the sexual cycles of particular species of the above parasites. Nevertheless, much has been learned from the examination of dissected preparations and sections of flies that had fed at known times on birds harboring different species of *Leucocytozoon* and *Haemoproteus*, although the history of the flies prior to these feedings was unknown. Observations of several kinds of ornithophilic flies harboring different species of these parasites are presented herein to clarify our concept of sporogony in the two genera and modify previous conclusions regarding *Leucocytozoon*. For example *Simulium venustum* rarely feeds naturally on ducks (4) and in our earlier work (13) *S. rugglesi* was almost certainly mistaken for it as the latter is known to be an important host of *L. simondi* (10). Comparisons of these parasites with other Sporozoa suggest relationships on the basis of which some revisions in classification are suggested.

[1]Manuscript received January 7, 1961.
Contribution from the Department of Parasitology, Ontario Research Foundation, Toronto, Ontario. This research was supported by a grant received by the Ontario Research Foundation from the Province of Ontario, through the Department of Planning and Development.

[The previous number of Can. J. Zoology (**39**, 123–213) was issued April 19, 1961.]

Materials and Methods

Specimens of various simuliids and ceratopogonids were collected after they had fed on different birds infected with species of *Leucocytozoon* and *Haemoproteus* respectively. Some of these insects referred to in the text as single species are undoubtedly complexes. For example, *Simulium aureum* is a cytogenetic complex of two or more species (9) that have yet to be resolved. Similarly *latipes* is probably a complex as well as the species of *Culicoides* near *piliferus*.

Species of the parasites in the different birds have been identified as follows; some revisions may be necessary following further studies of the life histories of various species.

Anas boschas (duck)	*L. simondi* Mathis & Leger
	H. nettionis (Johnston and Cleland)
Cyanocitta cristata (blue jay)	*L. sakharoffi* Sambon
Corvus brachyrhynchos (crow)	*L. sakharoffi*
	H. danilewskii Krüse
Bonasa umbellus (ruffed grouse)	*L. bonasae* Clarke
Zonotrichia albicollis (white-throated sparrow)	*L. fringillinarum* Woodcock
	H. fringillae (Labbé)
Quiscalus versicolor (bronzed grackle)	*L. fringillinarum*
Turdus migratorius (robin)	*L. mirandae* Franca
Aegolius acadica (saw-whet owl)	*L. danilewskyi* Ziemann
Canachites canadensis (spruce grouse)	*H. canachites* Fallis & Bennett
Carpodacus pupureus (purple finch)	*H. fringillae*
Padda oryzivorae (Java sparrow)	*H. oryzivorae* Anschütz

Most engorged flies recovered from birds were kept under field conditions where the temperature varied from 7° C to 26° C with a mean of approximately 16° C. A few that will be referred to specifically were kept at 22° ± 2° C. Most observations of sporogony were made on preparations of flies dissected in saline at intervals after they had ingested known parasites. Exflagellation of species of *Leucocytozoon* and *Haemoproteus* was observed when drops of blood containing gametocytes from different birds were added to citrated saline. In addition, dried and stained smears of the stomach contents of infected flies were examined. Sections were prepared from specimens fixed in Bles or Carnoy's solution, embedded in a high melting paraffin, and stained with Giemsa's stain or with haematoxylin and eosin.

Results

Microgamete Formation and Fertilization of the Macrogamete

Microgamete formation was followed most intensively for *L. simondi* and *H. oryzivorae* although it was seen and appears similar for *L. bonasae*, *L. sakharoffi*, *L. fringillinarum*, and *H. nettionis*. In saline preparations of *L. simondi* and *H. oryzivorae* held at 15°–25° C, some microgametes were produced within 3 minutes after gametocytes were withdrawn from a bird; others continued to appear for at least 20 minutes. Exflagellation of these organisms was more rapid than that reported for *Plasmodium gallinaceum* by Bishop and MacConachie (5).

Formation of microgametes of *Leucocytozoon* and *Haemoproteus*, as ascertained from examination of stained smears of citrated saline preparations and of the stomach contents of infected flies, appeared as follows. The mature

gametocytes, following ingestion by a simuliid, were liberated from the blood cells. The rather diffuse chromatin in the microgametocyte assumed a "spireme-like"* arrangement (Fig. 1) following which it appeared as a densely staining mass (Fig. 2). This solid mass broke up into several discrete particles (Fig. 3) each of which became incorporated in a strand of cytoplasm that was extruded and broke free from the surface of the gametocyte (Figs. 3, 4, 23) to become a microgamete (Figs. 5, 11). In contrast no change in the appearance of macrogametes was detected (Fig. 22). At least eight, and possibly more, gametes are produced by each microgametocyte. Some of the rapidly moving microgametes contact macrogametes (Fig. 12). Penetration of the microgamete into a macrogamete has not been observed but the appearance of fixed and stained preparations (Figs. 6, 7, 13) suggests that the entire microgamete enters. Polar bodies have not been observed. These could form and be overlooked because of the speed of exflagellation, but more probably reduction division occurs in the first nuclear division of the zygote in view of Stohler's (29) report of its occurrence at this point in the cycle of *Plasmodium gallinaceum* and Bano's work (3) on this and other species of *Plasmodium*.

Zygotes and Oökinetes

The zygotes of both *Leucocytozoon* and *Haemoproteus* stained so intensely that the nuclei were often obscured (Figs. 8, 24). One to four hours after a zygote formed a portion of it extruded rather like a pseudopodium (Fig. 9). The zygote continued to elongate, became vacuolated, and assumed the typical form of the oökinetes (Figs. 10, 25, 26, 27).

Oökinetes of *Leucocytozoon* and *Haemoproteus* will form in several genera of blood-sucking flies but complete sporogony has been found during the present study only in simuliids and *Culicoides* respectively. Oökinetes of *Haemoproteus* were about half the size of those of *Leucocytozoon* and contained "pigment granules" (Figs. 25, 26), although in some species most of these granules were "pinched off" from the oökinetes (Figs. 26, 27) before the oöcysts formed; a similar phenomenon has been reported by others (16, 22, 27, 31). Oökinetes of any one species of either *Leucocytozoon* or *Haemoproteus* may differ slightly in size and shape, and may contain a variable number of vacuoles. Only sluggish movement of the ends of oökinetes of both *Leucocytozoon* and *Haemoproteus* was seen, and even this was not always detected in saline preparations of the stomach contents of infected flies. This movement alone is unlikely sufficient to enable the oökinetes to penetrate the stomach wall of the fly. Possibly they move into the wall by a flowing, amoeboid movement. We have not seen an oökinete penetrating the stomach wall in any of our sections of simuliids or ceratopogonids as was illustrated by Stohler (29) for *P. gallinaceum* in *Aedes aegypti*.

Oökinetes were seen among the stomach contents of various simuliids 12–48 hours, and in some 72 or more hours, after the ingestion of gametocytes of *L. simondi*, *bonasae*, *fringillinarum*, *sakharoffi*, *mirandae*, and *danilewskyi*. Some of these oökinetes, although showing slight motility, may be moribund and will be eliminated in the fly's feces. Penetration of the stomach wall by others may be prevented until the peritrophic membrane has ruptured as reported for

*This is not necessarily an arrangement of chromosomes.

oökinetes of *Plasmodium circumflexum* (26) and *P. gallinaceum* (29). Such delay would account for some variability in the rate of sporogony that is referred to later. The delay may be more pronounced in some species of flies than others depending on the rate of digestion of their blood meals and their suitability as hosts for particular parasites. Limited observations on the oökinetes of *Haemoproteus* in *Culicoides* (12) gave little indication that the peritrophic membrane interfered with them. However, more attention to the membrane in ceratopogonids and simuliids relative to the penetration of oökinetes is desirable.

Oöcysts and Sporozoites

It was suggested formerly (13) that some oökinetes of *L. simondi* may change into oöcysts in the lumen of the stomach of infected flies since structures

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FIGS. 1–13, 18, 19, 21–27, 29–32 prepared from fixed and stained preparations, others from living specimens observed in flies dissected in 0.85% saline.

FIGS. 1–3. Changes in appearance of chromatin in nucleus of microgametocyte of *L. simondi* immediately before exflagellation. (1) "Spireme-like" arrangement of nucleus; (2) chromatin in solid mass; (3) chromatin divided into discrete particles.

FIG. 4. Microgametes of *L. simondi* extruding from gametocyte.

FIG. 5. Microgametes of *L. simondi*.

FIGS. 6–8. Developing zygotes of *L. simondi*: (6–7) microgametes have penetrated macrogametes but fusion of nuclei has not occurred; (8) zygote.

FIG. 9. Zygote of *L. simondi* beginning to elongate into oökinete.

FIG. 10. Oökinete of *L. sakharoffi* from stomach content of *Simulium aureum* that ingested gametocytes of *L. sakharoffi* 24 hours previously with blood from blue jay.

FIGS. 11–13. Gamete and zygote formation of *L. fringillinarum* as seen in fixed and stained citrated-saline preparation of blood from mourning warbler (*Oporornis philadelphia*): (11) microgamete; (12) microgamete about to penetrate (?) macrogamete; (13) microgamete has penetrated macrogamete.

FIG. 14. Oökinete of *L. sakharoffi* appears folded in oöcyst in wall of stomach of *Simulium aureum*.

FIG. 15. Oöcyst of *L. mirandae* appears as solid mass with vacuole (?) in stomach wall of *S. latipes*.

FIG. 16. Diagrammatic representation of oöcyst, presumably of *L. sakharoffi*, in stomach wall of *S. aureum*; sporozoites are forming and residual body is apparent.

FIG. 17. Oöcyst, presumably of *L. sakharoffi*, in stomach wall of *S. aureum*; sporozoites in characteristic arrangement prior to showing motility.

FIGS. 18–19. Sections of stomach wall of *S. rugglesi* containing oöcysts of *L. simondi*. Oöcysts appear to be intracellular; sporozoites cut transversely in one, longitudinally in the other, residual bodies distinct.

FIG. 20. Oöcyst of *L. simondi* in stomach wall of *S. rugglesi*, only a few sporozoites remain.

FIG. 21. Sporozoites, presumably of *L. sakharoffi*, from salivary gland of *S. aureum*.

FIG. 22. Macrogamete of *H. oryzivorae* 2 hours after blood, containing gametocytes from *Padda oryzivorae*, was mixed with citrated saline at 22° C.

FIG. 23. Microgamete formation of *H. fringillae* a few minutes after mixing infected blood from a white-throated sparrow with citrated saline.

FIG. 24. Zygote of *H. fringillae* from stomach content of *Culicoides sphagnumensis* 56 hours after ingesting blood from an infected purple finch.

FIG. 25. Oökinete of *H. fringillae* from stomach content of *C. sphagnumensis* 20 hours after ingesting blood from infected purple finch.

FIG. 26. Oökinete of *H. fringillae* in which most of "pigment granules" appear to be detaching from the oökinete; from stomach contents of *C. sphagnumensis* 20 hours after ingesting blood from an infected white-throated sparrow.

FIG. 27. Oökinete of *H. nettionis* which lacks "pigment granules"; from stomach contents of *Culicoides* near *piliferus* 40 hours after ingesting blood from an infected duck.

FIG. 28. Oöcyst, presumably of *H. nettionis*, in stomach wall of *Culicoides* near *piliferus* that fed on infected duck 10 days previously.

FIGS. 29–30. Sporozoites, presumably of *H. nettionis* and *H. danilewskii*, from *Culicoides* near *piliferus* and *C. crepuscularis* respectively.

PLATE I

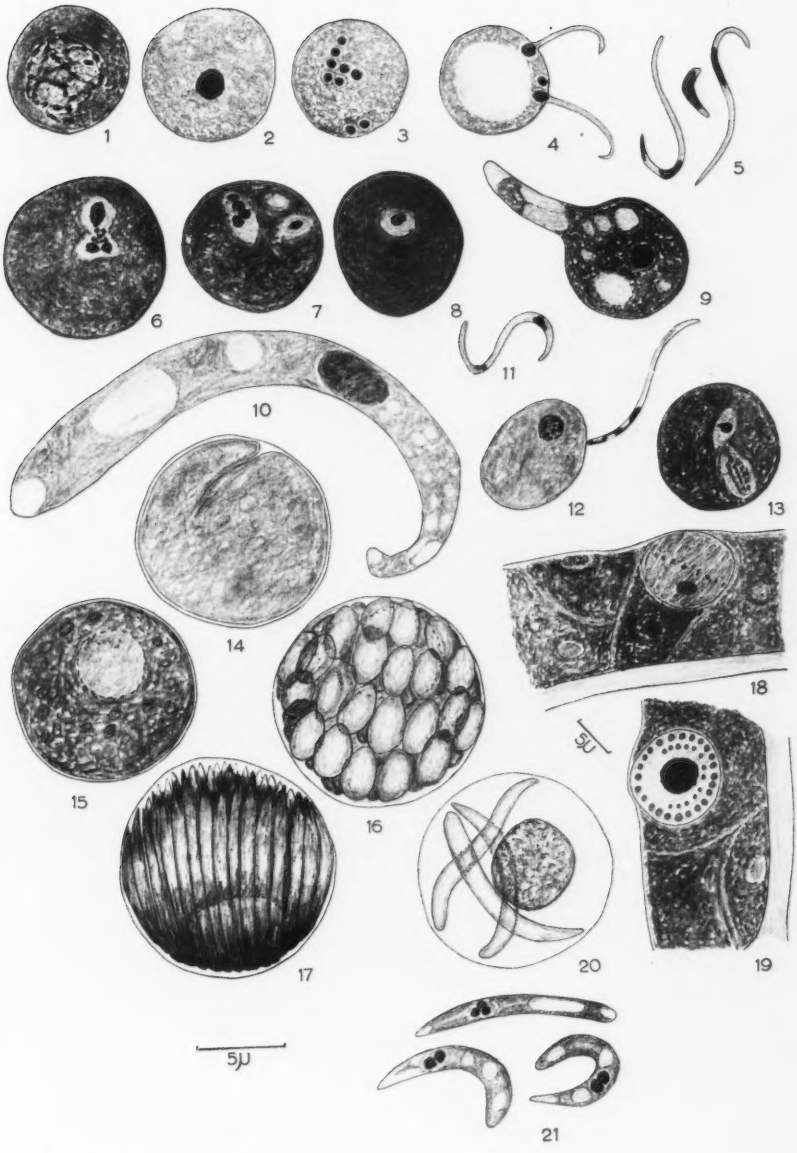

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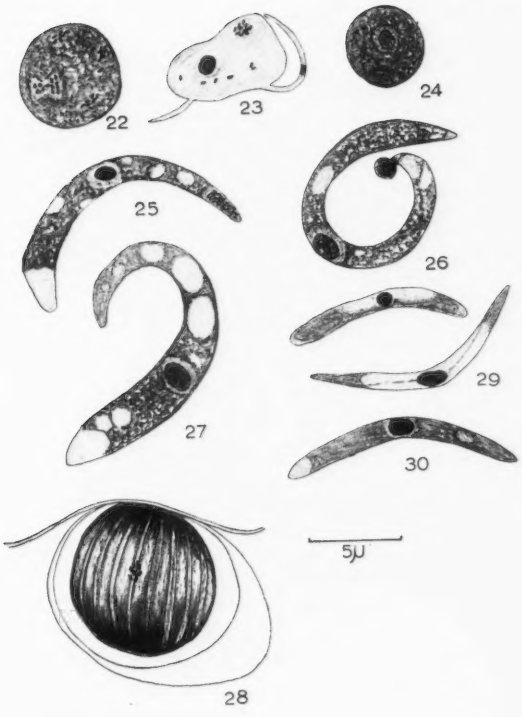
PLATE II



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thought to be developing oöcysts were found in smears made from dissected preparations of infected flies. These structures were found in a few simuliids that ingested gametocytes, of *L. simondi* and *L. bonasae* respectively, 40–48 hours previously. Twenty-five of these structures (Fig. 31), averaging 9.6 (6–12) μ with vacuoles 3–5 μ in diameter, contained 2–33 discrete chromatin masses. The size and appearance of these structures lead us to believe they are oöcysts which were torn from the stomach walls of the flies during the dissections, rather than individuals that were developing in the lumen of the stomach.

Examination of dissected and sectioned specimens of *S. rugglesi* containing developing stages of *L. simondi* revealed oöcysts that appeared to be in, as well as on, the stomach wall (Figs. 18, 19, 32). Moreover some appeared to be intracellular. Possibly they occur in both positions and when approaching maturity are located below the membrane surrounding the stomach of the flies. Development of oöcysts of different species of *Leucocytozoon* as seen in whole mounts of stomachs of *S. rugglesi*, *S. aureum*, *S. latipes*, and *Prosimulium decemarticulatum*, *Cnephia* "U" appeared as follows. Some parasites in early stages of development were folded within a cyst (Fig. 14), others appeared as a solid mass with a vacuole (Fig. 15) but, most probably, division of the chromatin in some of them had begun. Division of the cytoplasm (Fig. 16) to form sporozoites followed, and a residual body seemed to take the place of the vacuole. Presumably fragmentation of the chromatin preceded this development (Fig. 31) although this has not been detected in unstained preparations. Development continued and the sporozoites became arranged around the residual body (Fig. 17) somewhat like those illustrated for *Lankesterella* (24) and *Haemoproteus* (12). As the oöcyst matured the sporozoites became motile and intertwined. Oöcysts of *Leucocytozoon* are only slightly larger than zygotes; those of various species measured 10–15 μ in diameter and contained residual bodies 3–6 μ in diameter (Table I).

Oöcysts of *H. nettionis* (Figs. 28, 34, 35), like those of *H. canachites* (12), measured less than 10 μ and had few "pigment granules" in the residual bodies (3–6 μ). Occasionally in the dissection of the stomach from an infected fly the membrane surrounding some parasites was ruptured and the developing sporozoites were spread out around the portion of parasite regarded as residual body (Fig. 33). Developing oöcysts of *Haemoproteus* were observed also in the walls of the stomachs of *C. crepuscularis* and *C. stilobezzioides* that, 8 days previously, had ingested gametocytes of *H. fringillae* from the purple finch. Sporozoites of *Haemoproteus* were found in specimens of *C. crepuscularis* and *C. sphagnumensis* that fed 7 or more days previously on infected crows and purple finches; however, further transmission studies are required to determine the importance of these *Culicoides* as hosts for these and other species of *Haemoproteus*.

The small size of oöcysts of *Leucocytozoon* and *Haemoproteus* is in contrast to that reported for species of *Plasmodium* (18, 20, 28) and to that given by Adie (1) and Mohammed (23) for *H. columbae*. Mature oöcysts of *Leucocytozoon* contained an estimated 30–50 sporozoites and those of *Haemoproteus* fewer than 30. Oöcysts of *Leucocytozoon* containing fewer than 10 sporozoites (Fig. 20) as well as many containing only residual bodies were noted. Similar

observations were reported for *H. canachites* in *Culicoides*. Oöcysts with clearly ruptured walls were not seen. These observations lead to the conclusion that sporozoites of *Leucocytozoon* and *Haemoproteus* escape gradually without rupturing the wall of the oöcyst.

Sporozoites of *Leucocytozoon* (Fig. 21) measure 9–13 μ and are more pointed at one end. Some are slightly longer and narrower than others; possibly this is a specific difference. Sporozoites of *Haemoproteus* in our material are longer (10–14 μ) than those of *Leucocytozoon* and the ends are more uniformly rounded or pointed (Figs. 29, 30). Those of *Haemoproteus* illustrated by Adie (1), O'Roke (25), and Mohammed (23) from hippoboscids are smaller and one end tends to be blunt.

Rate of Sporogony

An indication of the rate of sporogony of different species of *Leucocytozoon* was obtained from dissections of flies at intervals following their ingestion of parasites from various birds. The method has limitations as most of the flies were kept under field conditions where the temperature varied from 7–26° C (av. 16° C), although some were kept at 22° ± 2° C. Presumably most of these flies had not fed on infected birds except at the time they were collected as (i) they were captured between May 30 and June 2 at the beginning of the "fly" season, (ii) oöcysts and sporozoites were not found in any simuliids, except one, that were collected at the same time after feeding on non-infected birds (Table II). Oöcysts were noted (Table II) in *P. decemarticulatum*, *S. latipes*, *S. aureum*, and *S. croxtoni* approximately 64 hours after these flies ingested gametocytes of *L. fringillinarum*. Sporozoites were found in the salivary glands of the same species of flies and in *S. aureum* and *Cnephia* "U" $3\frac{1}{2}$ to 5 days after they had fed on birds infected with *fringillinarum*. A similar pattern of development was noted in flies that ingested gametocytes of *L. bonasae* and *mirandae*. Neither oöcysts nor sporozoites were found in any of the flies examined 1 to 2 days after they had fed on infected birds, nor, except for a single *Cnephia*, in samples of "control" flies fed at the same time on non-infected birds. It is concluded therefore that the oöcysts and sporozoites were those of *L. fringillinarum*, *bonasae*, and *mirandae* respectively and that sporogony of some individuals requires less than 5 days. To obtain a measure of the slowest rate of sporogony, other flies were dissected at longer intervals following the ingestion of parasites. Specimens of *S. aureum* that fed 18 days previously on crows infected with *L. sakharoffi* contained oöcysts with sporozoites, and others which were empty except for residual bodies. Specimens of *S. rugglesi* that ingested gametocytes of *L. simondi* 16 days previously contained a few oöcysts with sporozoites, although the majority were empty except for residual bodies. Oöcysts were noticed also in specimens of *S. latipes*, *S. aureum*, and *P. decemarticulatum* 10–14 days after feeding on various birds. Limited observations on rate of sporogony of *Haemoproteus* in *Culicoides* indicate variation also (12) although it was more rapid than that reported for *H. columbae* in hippoboscids (1).

Comparisons and Classification of Haemosporidiida

Comparisons of *Leucocytozoon*, *Haemoproteus*, and *Plasmodium* (Table III)

PLATE III

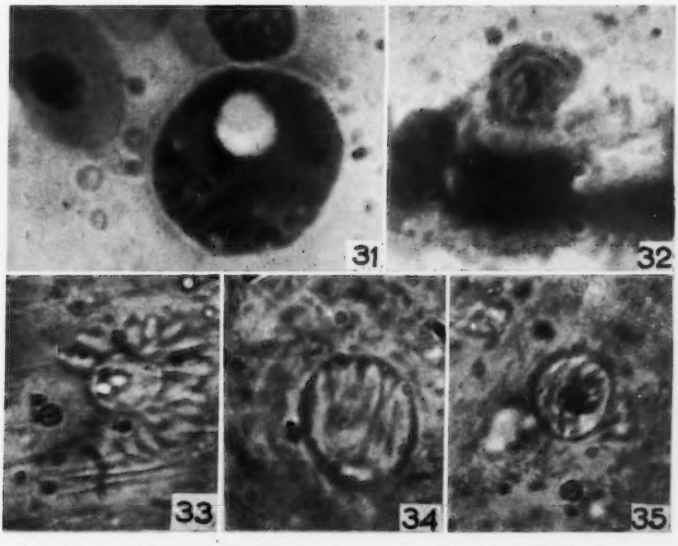

FIG. 31. Oöcyst of *L. simondi* in which chromatin has begun to divide.
FIG. 32. From section through oöcyst of *L. simondi* on stomach wall of *S. rugglesi.*
FIG. 33. Developing sporozoites of *H. canachites* as seen when oöcyst was ruptured during dissection of an infected specimen of *Culicoides.*
FIG. 34. Oöcyst with sporozoites of *H. canachites* on stomach wall of *C. sphagnumensis.*
FIG. 35. Oöcyst with sporozoites of *H. nettionis* on stomach wall of *Culicoides* near *piliferus.*

TABLE I

Diameter of oöcysts of *Leucocytozoon* in different simuliids

Species			Oöcyst		
Bird	Parasite	Fly	No. measured	Diameter, μ	Residual body, μ
Duck	*simondi*	*rugglesi*	25	10–14 (11.5)	3–6
Crow	*sakharoffi*	*latipes*	10	10–12 (11)	
Robin	*mirandae*	*latipes*	8	11–15 (12.5)	4–6
Robin	*mirandae*	*decemarticulatum*	15	9–13 (11.4)	3–6
White-throated sparrow	*fringillinarum*	*latipes*	1	12	
White-throated sparrow	*fringillinarum*	*aureum*	2	12	
White-throated sparrow	*fringillinarum*	*decemarticulatum*	4	10–13 (10.7)	
Grackle	*fringillinarum*	*Cnephia* "U"	14	10–13 (11.3)	4–5

TABLE II

Results of dissections of simuliids 1 to 6 days after feeding (May 30 and June 2) on birds with, and without, infections with *Leucocytozoon*

Parasite and host	Simuliids dissected: Days after feeding	Species	No.	Number with: Oöcysts	Sporozoites	Remarks
L. bonasae in *Bonasa umbellus*	<1	*latipes*	2	0	0	These flies were kept at an average daily temperature of 16° C
	2½–3	*latipes*	2	2	0	
	2½–3	*aureum*	1	1	0	
	2½–3	*croxtoni*	1	1	0	
	4½–5	*latipes*	37	No exam.	37	
L. fringillinarum in *Quiscalus versicolor*	4½–5½	*latipes*	14	14	14	
	4½–5½	*decemarticulatum*	4	4	4	
	4½–5½	*Cnephia* "U"	7	7	7	
	5½	*aureum*	1	1	1	
L. mirandae in *Turdus migratorius*	4½–5½	*latipes*	2	2	2	
	4½–5½	*Cnephia* "U"	4	4	4	
	5½	*aureum*	1	1	1	
	5½	*decemarticulatum*	1	1	1	
Various uninfected birds	4½–5½	Neither oöcysts nor sporozoites in 15 *latipes*, 2 *aureum*, 2 *decemarticulatum*, and 9 of 10 *Cnephia* "U"				

TABLE II (*Concluded*)

Results of dissections of simuliids 1 to 6 days after feeding (May 30 and June 2) on birds with, and without, infections with *Leucocytozoon* (*Concluded*)

L. fringillinarum in *Quiscalus versicolor*	1 –1½	*decemarticulatum*	5	0	0	
	1½	*aureum*	1	0	0	
	2½	*decemarticulatum*	2	2	0	
	2½	*aureum*	1	1	0	
	2½	*croxtoni*	1	1	0	
	2½–3½	*latipes*	3	3	0	
	3½	*croxtoni*	1	1	1	
	3½	*decemarticulatum*	1	1	1	These flies were kept in incubator at 22° ± 2° C
	4½–5	*decemarticulatum*	32	No exam.	31	
	4½–5	*latipes*	9	No exam.	8	
	4½–5	*aureum*	8	No exam.	6	
	4½–5	*croxtoni*	2	No exam.	2	
	4½–5	*Cnephia* "U"	1	No exam.	1	
L. fringillinarum in *Zonotrichia albicollis*	4½–5	*decemarticulatum*	9	No exam.	9	
	4½–5	*latipes*	4	No exam.	4	
	4½–5	*aureum*	7	No exam.	7	
L. mirandae in *Turdus migratorius*	1 –1½	*decemarticulatum*	4	0	0	
	3½–4	*latipes*	1	1	0	
	4½–5	*latipes*	19	No exam.	15	
	4½–5	*aureum*	1	No exam.	1	
	4½–5	*croxtoni*	4	No exam.	4	
Various uninfected birds	2½–5	Neither oöcysts nor sporozoites in 12 *decemarticulatum*, 8 *latipes*, 37 *aureum*, 2 *croxtoni*				

TABLE III

Comparisons of the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon**

	Plasmodium	*Haemoproteus*	*Leucocytozoon*
Sporogony*	Mosquitoes 1–3 weeks	Ceratopogonids 6–7 days Hippoboscids 1–2 weeks	Simuliids 5–6 days
Exflagellation	Rapid	Very rapid	Very rapid
Oöcysts	Large	Small in ceratopogonids. Large in hippoboscids	Small
Oöcyst wall	Ruptures	Collapses	Collapses
Sporozoites	Many; ends pointed	Few; ends pointed in ceratopogonids, one end less so in hippoboscids	Few; one end more pointed than other
Residual body	Present	Present	Present
Schizogony	In peripheral blood and certain tissues only	In certain tissues only	Widespread in various tissues
Asexual trophozoites	In peripheral blood and tissues	In tissues, scarce in blood	In tissues, scarce in blood
Prepatent period*	5–11 days	2–3 weeks	5–10 days
Gametocytes	In erythrocytes; pigment; may or may not distort host cell; nucleus of host cell may, or may not, be displaced	In erythrocytes; pigment; host cell often slightly enlarged; nucleus of host cell may, or may not, be displaced	In erythrocytes and leucocytes; No pigment; host cell distorted; nucleus of host cell distorted and displaced
Time for gametocytes to mature	2–4 days	4–6 days	2–3 days

*Some statements in the table are rather general as it is well known that rate of sporogony of *Plasmodium* varies with the species as well as with the temperature and the prepatent period is not the same for all species.

permit certain generalizations concerning their relationships within the Sporozoa. Sporogony in all three genera occurs in Diptera. Rapid exflagellation occurs in each although it is slower in *Plasmodium*. Similar numbers of microgametes are produced by each. Development of the oöcysts is more rapid in *Leucocytozoon* and *Haemoproteus* (except those in Hippoboscidae) than in *Plasmodium*. Oöcysts of *Haemoproteus* (12) (except those reported in Hippoboscidae (1, 23)) and *Leucocytozoon* are only slightly larger than the zygotes from which they arose whereas those of *Plasmodium* are much larger (18, 28). The capsule around the oöcysts of the two former may persist for some time after the escape of the sporozoites. Mayne (21) remarked on a similar condition in *Plasmodium* but Mackerras and Ercole (20) noted a rapid disappearance of the cysts after they had burst and the sporozoites escaped. Bursting of the oöcysts of *Leucocytozoon* and *Haemoproteus* and release of their sporozoites, as described for *Plasmodium* (20), has not been seen in the present study but the presence of oöcysts containing only a few remaining sporozoites suggests a gradual escape. Hundreds of sporozoites are produced in oöcysts of *Plasmodium* but fewer than 50 in species of *Leucocytozoon* and *Haemoproteus* we have studied. Sporozoites of the latter resemble somewhat those of *Plasmodium*; those of *Leucocytozoon*, having one end more pointed than the other, resemble some of those illustrated for haemogregarines.

An exoerythrocytic cycle is found in all genera but the schizonts of *Haemoproteus* and *Plasmodium* develop in more restricted sites than those of *Leucocytozoon*. The megaloschizonts of the latter are intercellular according to Cowan (8) although others regard them as intracellular (13, 17). The prepatent period reported for infections with *Haemoproteus* is longer than that for infections with *Leucocytozoon* and *Plasmodium*. Asexual stages are scarce in the peripheral blood of birds infected with *Leucocytozoon* and *Haemoproteus* although at times some are present as artificial transmissions of the parasite are reported following the transfer of blood from infected to non-infected birds (10, 19). This conclusion is not accepted by some, who maintain that merozoites transferred by the blood inoculation develop into gametocytes only, since no tissue stages have been demonstrated in the recipient birds. Undoubtedly this criticism is justified in some instances. Failure to demonstrate tissue forms is not proof of their absence, however, for schizonts, especially of *Leucocytozoon*, develop in many tissues and, except in heavy infections, are not easily demonstrated even in those initiated with sporozoites. Moreover, as reported previously (10), infections with *Leucocytozoon* were produced with blood removed from birds 2 to 3 days before any developing gametocytes could be demonstrated in their blood. Furthermore, gametocytes were not apparent in some recipient birds until several days after they were inoculated. This would be expected if the gametocytes resulted from an asexual cycle initiated by parasites transferred in the inoculum of blood. Such a cycle is suggested also by the increasing parasitaemia noted in some birds inoculated with blood. Parasites in all three genera invade erythroblastic cells and *Leucocytozoon* invades leucocytes also. No pigment is produced by *Leucocytozoon*. In some species of *Haemoproteus* much of the pigment is "pinched off" from the oökinetes before the oöcysts are formed but this is not reported for *Plasmodium*. Chronic infections persist for long periods and relapses occur

which are more pronounced in infections with *Plasmodium* than with the others, *Leucocytozoon* and *Haemoproteus* with small oöcysts (as seen in *Culicoides*). having a rather resistant membrane, absence of sporocysts and few sporozoites, resemble *Haemogregarina*, *Lankesterella*, and *Legerella*. The life cycles of *Haemogregarina* and *Lankesterella* require an intermediate host although apparently this is a transfer host only for *Lankesterella*. No such host is required for *Legerella*, which is perhaps indicative of the primitive condition. *Leucocytozoon* is probably more primitive than *Haemoproteus* and *Plasmodium* as it has (i) two types of schizonts, one of which occurs in any tissue and produces many merozoites, (ii) a short prepatent period, and (iii) gametocytes that develop in more than one type of blood cell and do not produce pigment. The present observations on *Leucocytozoon* and *Haemoproteus* appear therefore to support the view, advanced some time ago by Reichenow and Doflein (as quoted by Wenyon) (32), that *Leucocytozoon* is closely related to the haemogregarines. Bray (6) also develops the thesis that the Haemosporidiida have evolved from the haemogregarines of the Adeleida. In his view the Haemoproteidae and Plasmodiidae should be retained as families and two or perhaps three genera should be formed in the Plasmodiidae. Corradetti (7) would place all three genera in the Plasmodiidae. Garnham (15) suggested placing *Leucocytozoon* in a separate suborder. It seems to us that the similarities between *Leucocytozoon* and *Haemoproteus* are sufficient to retain them in the same order. However, the differences among these genera and *Plasmodium* (Table III) would justify placing each in a separate family, although the sporogony of certain species of *Haemoproteus* in hippoboscids and others in *Culicoides* raises a problem. Development of *Haemoproteus* in hippoboscids resembles that for *Plasmodium* in mosquitoes whereas that observed for species that develop in *Culicoides* is similar to *Leucocytozoon*. Possibly two different genera of parasites exist with similar gametocytes. It will be of interest, therefore, to discover whether sporogony of *H. columbae* and *H. lophortyx* can take place in *Culicoides*. If this is impossible, then the species of *Haemoproteus* with which we are working should be placed in a different genus.* In the meantime the following classification is proposed although perhaps, as Dr. Garnham has pointed out to us, the order Babesiida should be removed.

CLASS: Sporozoa
SUBCLASSES: I. Gregarinina
II. Coccidiomorpha

ORDER: I. Coccidiida
II. Adeleida
III. Babesiida
IV. Haemosporidiida

FAMILY: I. Plasmodiidae
II. Haemoproteidae
III. Leucocytozoidae

*Other interesting questions are raised by the recent report of Akiba (Japan. J. Vet. Sci. 22, 309–319, 1960) that *Culicoides arakawae* is a suitable host for *Leucocytozoon caulleryi*.

Perhaps it is more than coincidence that the most primitive of the three types of parasites under discussion develops in simuliids and the most specialized in culicids. The specificity of these parasites for particular groups of Diptera suggests they were initially parasites of insects. Walton (30) postulates that the Haemosporidiida were derived from a coccidia-like parasite in an invertebrate, the haemogregarines of invertebrates adopting cold-blooded vertebrates as alternate hosts, eventually alternating between an invertebrate and a warm-blooded host. The opposite condition presumably prevails in filarioid nematodes, which show more specificity for the vertebrate than for the invertebrate host, the former presumably being the ancestral type (2).

Acknowledgments

We are grateful to the Department of Lands and Forests of Ontario for the use of its laboratory facilities in Algonquin Park and wish to thank especially Mr. R. Standfield, the Director of this field station. We are indebted also to Dr. P. C. C. Garnham, Professor of Protozoology, London School of Hygiene, for reading the manuscript and making several suggestions.

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MORTALITY OF WINTER EGGS OF THE EUROPEAN RED MITE PANONYCHUS ULMI (KOCH), AT LOW TEMPERATURES, AND ITS ECOLOGICAL SIGNIFICANCE[1]

A. W. MacPhee

Abstract

Winter eggs of the phytophagous mite *Panonychus ulmi* (Koch) from Nova Scotia were killed by higher temperatures than were eggs from colder areas in New Brunswick and Quebec. For short exposures the mean lethal temperatures were respectively −24° F and −35° F. In the lethal temperature range the mortality of both forms increased about 10% for each doubling of the exposure period in the range from 15 minutes to 16 hours. Reciprocal crosses of the two forms indicated that the factor for resistance to cold in *P. ulmi* is recessive. Resistance was increased in the laboratory by selection. A method of estimating the winter mortality from weather records is outlined.

Introduction

There is little precise information on mortalities of overwintering stages of arthropods in relation to intensity of the cold and duration of the exposure. This is a report on mortalities and freezing points of overwintering eggs of the European red mite, *Panonychus ulmi* (Koch), from Nova Scotia, from colder areas in New Brunswick, and from a number of other areas.

The investigation was part of a general study on the biotic and physical factors that regulate the numbers of various arthropods. One of the most serious orchard pests, *P. ulmi*, has been studied extensively. The species has five to seven generations a year and increases very rapidly in the absence of natural enemies or other controls. It is now a pest throughout the temperate zone and was probably introduced to America from Europe. It was first observed in Nova Scotia in 1913 by Gilliatt (5). It overwinters as an egg which is different in several respects from the summer egg. The winter egg has a facultative diapause induced mainly by short day length (6).

Some arthropods survive low temperature by supercooling, but are killed on freezing whereas others survive freezing; *P. ulmi* belong to the first group.

Methods

This study was conducted largely in the laboratory and was supplemented by field observations. In the field *P. ulmi* was sampled for natural mortality by determining percentages hatching before and after cold periods. In the laboratory temperatures as low as −50° F were used. They were measured to the nearest half degree Fahrenheit with a manual or a recording potentiometer with copper constantan thermocouples made from 40-gauge wire. The points were either twisted together and soldered or fused together in a small carbon arc.

To determine percentage mortality ("group method") samples of 200 eggs

[1]Manuscript received January 30, 1961.
Contribution No. 1061 from the Research Station, Canada Agriculture, Kentville, Nova Scotia; from a thesis submitted in partial fulfillment of the requirements for a Ph.D. degree Department of Entomology and Plant Pathology, McGill University, Montreal, Que.

each were exposed *in situ* on the twigs for 0.5 to 32 hours. The temperatures of exposure were those covering the estimated range of lethal temperatures. The eggs were then incubated and those that were red and had substance showing within the shells were counted as unhatched. As some untreated eggs failed to hatch, mortality was calculated by Abbott's formula (1).

To determine freezing points, eggs were placed singly in contact with a thermocouple point and the temperature was lowered gradually from +34° F to the expected freezing temperature in 1 hour. The heat liberated when freezing occurred caused a sharp temporary rise in temperature. The lowest temperature reached when freezing occurred was taken as the freezing point. This method ("freezing point") was also used to study the effect of the duration of exposure by holding the temperature at a predetermined level and noting the interval between the end of the cooling period and the freezing of the egg.

In an experiment to determine the adaptability of *P. ulmi* populations with respect to cold hardiness, winter eggs from a Nova Scotia orchard were exposed for 2 hours at −24° F. The survivors were reared during the summer and the winter eggs produced in the fall were exposed to −25° F. This procedure was repeated at −26° F and −29° F in the third and fourth seasons.

Offspring of reciprocal crosses of resistant and susceptible forms were obtained by placing small groups of female deutonymphs of one form and males of the other on leaves or twigs in an incubator. A photoperiod of 8 hours of light and 16 hours of darkness (6) was used to initiate winter egg production.

Results and Discussion

Group Tests on Eggs from Various Areas

The results in Table I show that cold-hardiness of *P. ulmi* varied greatly between areas. The eggs collected in New Brunswick and Quebec suffered little or no mortality at −26° F. Mortality was about 25% at −30° F for exposure periods of 32 hours and the mortality was over 95% at −35° F after 8 hours. The eggs from Nova Scotia, Ontario, and Virginia were much more susceptible, mortality approaching 100% after 4 hours' exposure at −26° F.

TABLE I

Percentages of *P. ulmi* winter eggs from various areas that did not hatch after incubation following exposure to −26° F for various periods*

	Collection areas				
Duration of exposure	Nova Scotia Wolfville 1957	New Brunswick Fredericton 1957	Quebec Rougemont 1957	Ontario Vineland Station 1957	W. Virginia U.S.A. Kearneysville 1956
Cooling period (1 hr)	18	9	0	71	71
C.P. + ½ hr	78	0	7	88	100
C.P. + 1 hr	97	0	1	100	89
C.P. + 2 hr	95	25	5	94	98
C.P. + 4 hr	100	9	12	100	—
C.P. + 8 hr	99	17	6	99	—
C.P. +16 hr	100	32	5	—	—
C.P. +32 hr	100	23	32	—	—

*Percentages adjusted by Abbott's (1) formula.

TABLE II

Lowest temperatures (F°) 1947–1956,* for areas considered in study of *P. ulmi*

Place	1947	1948	1949	1950	1951	1952	1953	1954	1955	1956
Annapolis Royal, N.S. (near Deep Brook)	−1	−8	−2	−5	−7	0	7	−6	−1	−2
Kentville, N.S. (near Wolfville)	−5	−20	−6	−15	−22	−7	3	−13	−11	−7
Fredericton, N.B. (Exptl. Farm) (near Douglas and Gagetown)	−26	−32	−22	−22	−22	−21	−7	−17	−27	−12
Brome, Que. 1947–50 Farnham, Que. 1951–56 (both near Rougemont, Que.)	−26	−36	−20	−24	−31	−28	−15	−30	−32	−27
Grimsby, Ont. 1947–48 St. Catharines, Ont. 1949–1956 (both near Vineland Station)	0	−10	3	−2	0	2	10	0	−2	4
Morden, Man. (Exptl. Farm)	−28	−35	−40	−39	−35	−28	−24	−34	−30	−31

*From Climatic Summaries, Meteorological Division, Department of Transport, Canada.

The differences in susceptibility to cold were associated with differences (Table II) in the annual extreme low temperatures of the area concerned. Comparisons of mite cold-hardiness (on the basis of the "group tests") and low temperature of the area suggests that *P. ulmi* populations increase in resistance to cold as a result of being exposed to low temperatures which killed the more susceptible part of the population. An analysis of variance of the results of groups tests at −22° F, at −26° F, and at −30° F was carried out on percentage mortality transformed to arc–sine angles. The linear effect of duration of exposure on mortality was eliminated from the sum of squares for error in the variance table. The results showed that mortality increased with the duration of exposure, and that areas differed. The data were not sufficiently accurate to give quantitative values, to the effect of duration of exposure.

Freezing Points from Localities in Nova Scotia and New Brunswick

The freezing points of individual winter eggs from a number of localities in Nova Scotia and New Brunswick are shown in Table III. These data show the distinct difference between Nova Scotia and New Brunswick eggs, and show that there are discrete normal distributions of susceptible and resistant groups; also the resistant eggs (below −30° F) from the colder localities of Nova Scotia are similar in distribution and mean to those from New Brunswick. This shows the presence of two forms in the population as distinct from a continuum in cold-hardiness.

The collection locality having the least extreme low temperature was Deep Brook (near Annapolis Royal) where −8° F was the lowest temperature experienced in the 10-year (1947–56) period (Table II). Wolfville temperatures (near Kentville) were not below −22° F. Moderately low temperatures occurred in the Aldershot area of Nova Scotia where −26° F was reached in 1957, and the coldest area sampled was New Brunswick where −32° F occurred at Fredericton once in the 10-year period. The mean freezing point of eggs from Deep Brook was −24.6° F, and only one egg out of 500 survived −29° F. Approximately the same mean and distribution of freezing points occurred in the Wolfville collection. The lower freezing points of the eggs from Aldershot (Table III) was associated with the lower temperatures experienced there and the lower mean freezing point was caused by the presence of about 15 percentage cold resistant eggs in the population. Because 95 percentage of the eggs from New Brunswick were of the cold resistant form the mean freezing point was lowered to −35° F. The percentage of eggs resistant in a population is therefore associated with the climatic low temperature of the area.

The data in Table III also shows that the mortality of cold susceptible eggs begins at −22° F and increases rapidly as the temperature goes down. Areas experiencing −22° F to −26° F are threshold; Aldershot, Nova Scotia (Table III), is an example of such areas. Records indicate temperatures are 4 or 5 Fahrenheit degrees lower at Aldershot than at Kentville, and Kentville shows temperatures down to −20° F or lower twice in a 10-year period (Table II). In January 1957 the low at Kentville was −22° F and the low at Aldershot about −26° F. The temperatures at Aldershot would therefore cause mortality in the susceptible part of the population and favor the increase of the cold resistant form. This effect has been sufficient to cause the cold-resistant form

TABLE III

Numbers of *P. ulmi* winter eggs from various areas that froze at temperatures from −20° to −39° F

Collection area	Temperature, °F																					No. in test	Mean freezing point, °F
	Above −20°	−20°	−21°	−22°	−23°	−24°	−25°	−26°	−27°	−28°	−29°	−30°	−31°	−32°	−33°	−34°	−35°	−36°	−37°	−38°	−39°		
Wolfville, N.S.	2			7	11	27	20	21	10	1	1											100	−24.8
Gaspereau, N.S.	2		2	3	13	11	18	26	18	3	1	1	1				1					100	−25.5
Aldershot, N.S.		1		4	3	10	23	18	12	7	6	1	1	2			1	3	8			100	−27.4
Coldbrook, N.S.			3	1	4	14	22	21	14	9	1	3		2	1			2	2	1		100	−26.7
Canning, N.S.	3	2	1	4	11	16	30	15	16	1					1							100	−25.0
Berwick, N.S.		1		2	10	21	22	26	11	5					1	1						100	−25.6
Deep Brook, N.S.	5	11	20	38	75	122	86	83	38	15	6		1									500	−24.6
Douglas, N.B.			1	1	1						1		3	5	2	14	19	26	11	9	7	100	−35.3
Gagetown, N.B.	1										4	3	3	4	8	14	12	13	12	21	5	100	−35.3

TABLE IV

Numbers (in 100 egg sample) of *P. ulmi* winter eggs frozen at low temperatures from −18° to −28° F; comparison of collection dates and comparison of testing dates, 1958–59

Date collected	Date tested	Temperature, °F											
		−18° and above	−19°	−20°	−21°	−22°	−23°	−24°	−25°	−26°	−27°	−28° and below	Mean freezing point
Dec.	Dec.	2				7	11	27	20	21	10	2	−24.8
Dec.	Feb.	1				4	11	15	30	19	15	5	−25.10
Jan.	Feb.		1		1	5	14	18	27	23	5	6	−25.21
Feb.	Feb.					4	7	16	19	30	14	10	−25.93
Dec.	Apr.			1	3	7	25	25	26	9	3	1	−24.32
Jan.	Apr.	1		2	5	8	16	23	28	14	2	1	−24.28
Feb.	Apr.			2	8	10	24	30	23	8	1	1	−24.00
Mar.	Apr.	1	2	2	3	16	14	28	17	17	1	1	−24.00
Apr. 3	Apr.	3	2	5	1	8	18	27	20	11	6	1	−24.03
Apr. 21	Apr. 22	1		4	6	17	26	30	13	2	1		−23.45

to increase to 15% of the population. A balanced polymorphic condition has apparently been reached between cold-susceptible and cold-resistant forms. If this is so the susceptible form must have some slight advantage to offset the occasional selective pressure of cold which favors the cold-resistant form.

Environmental and Other Conditioning Influences

The procedure of investigating the cold-hardiness of *P. ulmi* necessitated comparisons of eggs collected at different times, from different places, grown on different varieties of apples, with variable periods of exposure to natural winter temperature, and with variable periods of storage at +34° F. The effect of the environment on cold-hardiness was, therefore, appraised as far as possible by tests in which individual factors were considered while others were held constant.

The effect of date of collection and date of testing on the cold-hardiness of the winter eggs, collected in Wolfville, are shown in Table IV. Samples were collected each month from December to April and exposures were made of all collected up to the test dates. The results are very similar irrespective of date of collection or time stored before testing up until the end of March, after which cold-hardiness decreased appreciably.

The freezing points of samples of eggs from each of two apple varieties in three different orchards in Nova Scotia were determined. The mean freezing point for eggs from varieties Delicious and Cortland in Wolfville, were respectively −24.3° F and −24.4° F; from Cortland and Ben Davis in Aldershot −26.5° F and −26.4° F; and from Delicious and Spy in Morristown −24.8° F and −25.0° F. This data indicates that the variety of host has no effect on the cold-hardiness of *P. ulmi*.

The effect of long exposure at 0° F on the mortality of winter eggs was tested by using the group method. The number that failed to hatch increased appreciably after 30 days' exposure and after 60 days' exposure practically no hatching occurred (Table V). The duration of 0° F weather during the winter would almost certainly be much less than a total of 30 days in Nova Scotia

TABLE V

The effect of 0° F on *P. ulmi* egg winter mortality for various exposure times

Dates removed to +34° F	Days* exposed to 0° F	Replicate 1		Replicate 2		Av. % unhatched
		Sample	% unhatched (dead)	Sample	% unhatched (dead)	
No tmt. check	0	10	29.4	20	25.0	27
No tmt. check	0	6	34.8	14	34.8	35
Feb. 21	4	4	34.3	11	29.1	32
Feb. 26	9	7	31.1	15	28.3	30
Mar. 4	15	8	42.1	13	27.7	35
Mar. 11	22	9	40.7	12	33.5	37
Mar. 19	30	2	47.1	17	34.9	41
Mar. 28	39	5	68.6	16	66.5	67
Apr. 8	50	1	96.5	18	93.6	95
Apr. 18	60	3	97.6	19	97.4	97

*Number of days of exposure at 0° F before removal to 34° F chamber.
NOTE: Egg samples placed in cold room February 17, and incubated April 18 to May 1, 1958.

and, therefore, no mortality in the field is expected from this cause.

A test on the effect of prolonged storage at +7° F on the freezing points of eggs showed that these did not change appreciably for various intervals up to 58 days.

Tests on the effect of the rate of cooling on the freezing points of eggs did not indicate that this was an important factor providing the rate was not excessively rapid. The fastest rate tested was cooling from +34° F to −25° F in ½ hour.

Analysis of the variation of freezing points of random samples of eggs indicated that a mean difference of one degree Fahrenheit between 100-egg samples was the maximum which could be expected due to chance. The thermocouples were corrected only to within 0.5 Fahrenheit degree. This additional error must be considered when comparing means of samples tested with different thermocouple points, many of which were used in these studies.

It is concluded that there is very little variation within the so-called susceptible form of red mite with respect to cold-hardiness, and that the environmental conditioning of the eggs during the winter months has little or no influence on cold-hardiness.

The Development of Resistance to Low Temperature in a Cold-susceptible Form of P. ulmi

The strikingly greater cold-hardiness in *P. ulmi* from New Brunswick as compared to those from Nova Scotia is shown in Fig. 1. The results of tests on environmental factors described above did not explain the reasons for this difference. The differences in susceptibility, discussed above and shown in Fig. 1, suggest the possibility of the presence of a genetically different, more cold-resistant, form predominating in New Brunswick. An experiment to test this possibility was undertaken in 1956 on cold-susceptible Nova Scotia eggs from the Wolfville population. The method, described above, was the application of selective pressure, that is, the killing by exposure to cold of the most cold-susceptible part of the population, during four successive winters. The progressive increase in resistance is illustrated in Fig. 2. The group which survived −30° F increased from 0.6% in the initial population to 91% in the eggs of the fourth selected population, as shown in Fig. 2. A check made in 1958 and 1959 on the freezing points of eggs from the Wolfville orchard showed 6 eggs out of 1000 tested survived −30° F; 0.6% were therefore probably resistant at the start of the experiment.

The increase in resistance to cold in the *P. ulmi* population strongly supports the hypothesis that the observed difference in cold-hardiness between the New Brunswick and the Nova Scotia populations, and within the latter, results from an inheritable genetic factor; and that resistance in a population can increase rapidly and has probably done so in the field.

The results suggest that further increase in cold-hardiness beyond the level attained by the New Brunswick form is unlikely. This tentative conclusion is supported by the following. (1) The fact that not one of the many eggs tested from New Brunswick survived −39° F even though the population has undoubtedly experienced many exposures to extreme cold. (2) None of those selected survivors of low temperature in the laboratory survived −39° F.

(3) Salt (9) suggests the possibility that few living arthropods supercool more than 72 Fahrenheit degrees below the freezing point of their tissues. (4) The fact that *P. ulmi* has not been found in Morden, Manitoba, although much suitable host plant material has been transplanted there from areas where this mite is prevalent. The low temperatures of areas such as Morden (Table II) would be expected to kill even the most resistant mites found in these samples. (5) There may be some significance to the fact that it has been found impossible to date to supercool water droplets below −41° F (7).

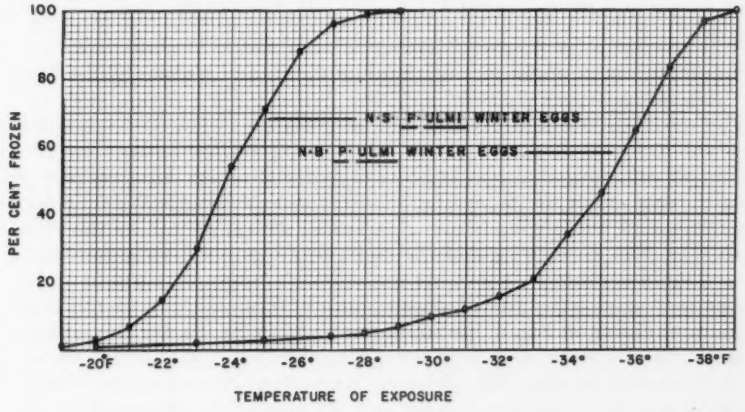

FIG. 1. Relation between percentage frozen and exposure temperature of *P. ulmi* winter eggs from Deep Brook, Nova Scotia, and from New Brunswick.

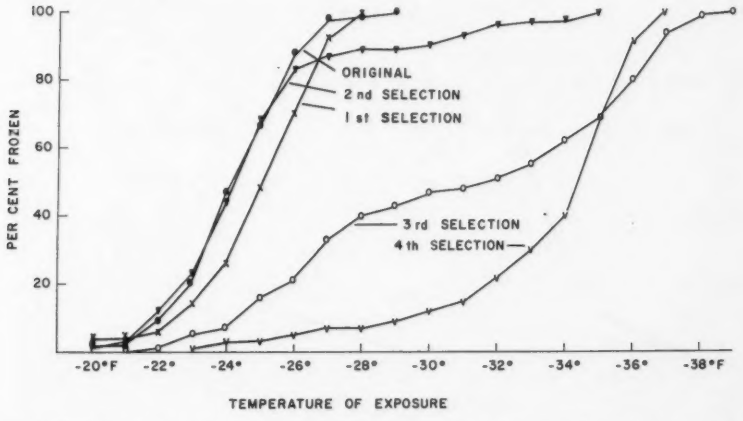

FIG. 2. Relation between percentage of *P. ulmi* winter eggs frozen and exposure temperature for an original population and for populations following one, two, three, and four annual exposures to low temperatures lethal to a part of the population.

This all suggests that −40° F may be approaching the lower limits of survival of those arthropods which are killed on freezing.

The distribution of the freezing points (Fig. 2) at each selection stage further suggests that the eggs either fall into a susceptible or a resistant category.

The development of increased cold-hardiness in an arthropod as means of survival is demonstrated in *P. ulmi*. The possibility of such occurrences has been suggested previously in a few instances: Ehrenhardt (4) on the basis of field observations reported some evidence of increased cold-hardiness in the woolly apple aphid, *Eriosoma lanigerum* (Hausm.); Batzer and Benjamin (2) showed evidence for this in the European pine shoot moth, *Rhyaciona buoliana* (Schiff).

The Cold-hardiness of the F_1 Generation of Reciprocal Crosses of Resistant and Susceptible Forms

The means and distribution of the freezing points of the susceptible and resistant forms of *P. ulmi* are now known within close limits. The group to which individual eggs belong can be identified with a high degree of probability. Crosses were made in September, 1959, of New Brunswick (Fredericton) females mated with Nova Scotia (Wolfville) males, as well as the reciprocal cross. The New Brunswick and the Nova Scotia population were respectively 94 and 0.6% resistant.

The response of the parent population and the offspring of the reciprocal crosses are shown in Fig. 3. The offspring of Nova Scotia females crossed with New Brunswick males were all in the susceptible group, that is, almost identical with the female parent. The offspring of the New Brunswick females crossed with Nova Scotia males were 43% in the susceptible group and 57% in the resistant group.

These results show sex-influence of the cold-hardiness factor and indicate that resistance is recessive. The fact that only 57% of the offspring of resistant

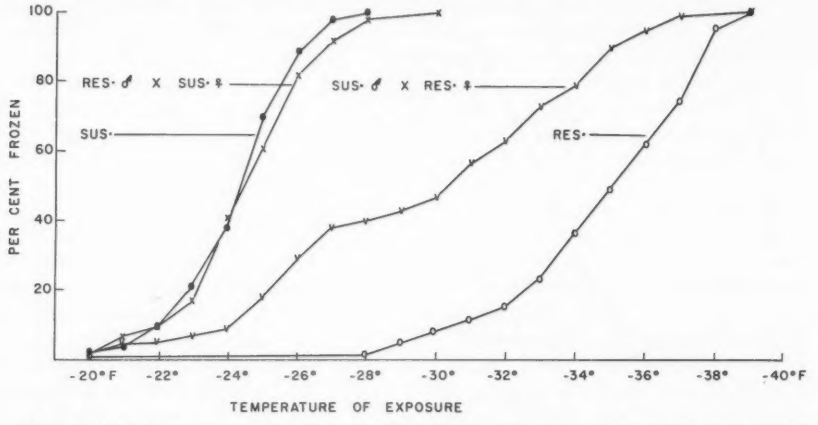

FIG. 3. Relation between percentage of *P. ulmi* winter eggs frozen and exposure temperature of susceptible (Wolfville, N.S.) and of resistant (central, N.B.) populations and of eggs from their reciprocal crosses.

females were resistant largely eliminates the possibility of cytoplasmic or maternal inheritance.

P. ulmi is a mite species in the same tribe (Tetranchini Reck) as *Tetranychus telarius* (L.) the males of which have been shown to be haploid by Schrader (10) and *P. ulmi* unmated females produce eggs which give rise to males only (3). This indicates that *P. ulmi* males are haploid and the character for resistance to cold is associated with any chromosome rather than specifically with a sex-determining chromosome.

Measurement of the Effect of Duration of Exposure

The effect of duration of exposure to low temperature on mortality was shown to be important by other investigators, for example by Salt (8), it therefore seemed desirable to measure this factor and to relate it to mortality. The situation was opportune because *P. ulmi* give precisely reproducible data with the freezing point method and because this method can be adapted to measure the effect of duration of exposure.

The freezing point method was used to obtain the data shown in Figs. 4 and 5 and in Table VI. The temperature of eggs placed on the thermocouple points was lowered gradually to the temperatures shown under "temperature of exposure" in Table VI, and maintained there for 16 hours. The percentage which had frozen at the end of the periods indicated is shown in Figs. 4 and 5. The periods recorded were doubled progressively from 15 minutes exposure to 16 hours to give a uniformly logarithmic increase. Six series were run on Nova Scotia (susceptible) eggs and five series on New Brunswick (resistant) eggs. The percentage frozen was found to increase over the vulnerable range in linear proportion to the logarithm of the duration of exposure. The regression of mortality (percentage frozen) against duration of exposure was calculated for each series and the slope *b* of each is shown in Table VI and the regression lines are plotted in Figs. 4 and 5. The correlation coefficient *r* (Table VI) shows a good fit for all data.

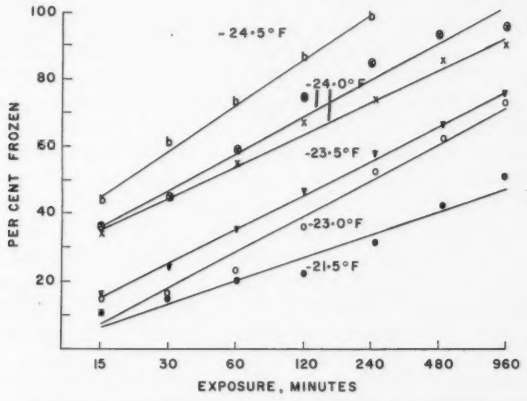

FIG. 4. Relation between percentage of Nova Scotia *P. ulmi* winter eggs frozen and time of exposure at the temperatures shown.

The slopes of the regression lines for areas and for temperatures are moderately uniform below −32° F for the resistant form and below −22° F for the susceptible form. At higher temperatures the slope *b* becomes less.

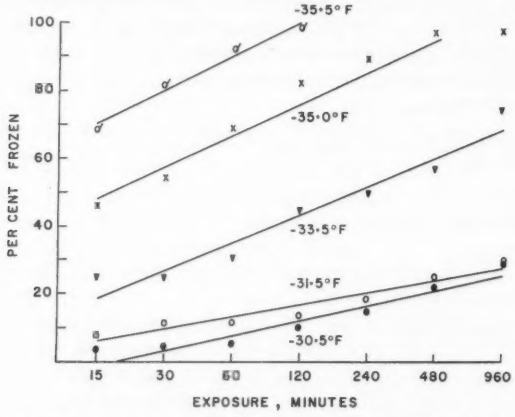

FIG. 5. Relation between percentage of New Brunswick *P. ulmi* winter eggs frozen and time of exposure at the temperatures shown.

TABLE VI

Data on the effect of time of exposure to various low temperatures on the mortality of *P. ulmi* for exposure periods up to 16 hours; test results shown graphically in Figs. 4 and 5 (series identified by temperature of exposure)

Place and year	Temperature of exposure, °F	No. of eggs in test	*b**	*r*†	Standard error of *b*
Wolfville, N.S. 1958	−21.5	99	22.1	.98	2.21
Deep Brook, N.S. 1959	−23.0	218	35.1	.98	3.12
Wolfville, N.S. 1959	−23.5	108	33.7	.99	0.56
Wolfville, N.S. 1959	−24.0	104	30.9	.99	1.49
Deep Brook, N.S. 1959	−24.0	134	35.5	.98	2.97
Deep Brook, N.S. 1959	−24.5	52	44.2	.99	1.71
Central, N.B. 1958	−30.5	97	14.1	.97	1.71
Central, N.B. 1959	−31.5	123	11.3	.96	1.49
Central, N.B. 1958	−33.5	113	27.4	.97	3.02
Central, N.B. 1959	−35.0	103	30.7	.97	3.13
Central, N.B. 1958	−35.5	143	33.7	.99	0.82

*b = linear regression coefficient of percentage mortality on logarithm of time of exposure.
†r = correlation coefficient.

The duration effect is equivalent to an increase in mortality of about 10% each time the duration of exposure is doubled after the initial 15 minutes' exposure. No attempt was made to investigate the effect of duration of exposure under 15 minutes. Extended periods of exposure over 16 hours have not been investigated; however, the lowest winter temperatures usually last only a few hours. The effect of temperatures in Nova Scotia between 0° F and −20° F have not been investigated in the laboratory but have not been observed to cause mortality in the field. Laboratory studies indicated mortality occurred only after over 30 days at 0° F.

These data show that duration of exposure is an important factor in low temperature mortality of *P. ulmi* and that a large error is to be expected if only the temperature of an exposure is considered in estimating mortality in the field. It follows that weather records should include the duration of low temperatures to be most useful for making estimates. The difference in mortality resulting from an exposure lasting 15 minutes compared to one lasting 4 hours might be as much as 40%.

Application of Results on the Effect of the Duration of Exposure

The expected mortality from cold weather in a winter population of *P. ulmi* can be estimated if the temperature and the duration of the cold period are known. One method is to estimate by inspection from the data in Figs. 4 and 5. Another method is to calculate the mortality from Fig. 1 and the *b* values in Table VI. The advantage of this method is that data in Fig. 1 is based on large numbers of eggs. The curve approximates the mortality from exposure during the cooling period plus 15 minutes' additional exposure at that temperature. The increase in mortality due to exposure over the 15 minutes can be calculated with the straight line formula:

$Y = a + bx$ (where Y = % mortality),

a = constant (calculated at 15 minutes' exposure for temperature concerned from Fig. 1),

b = slope of regression of percentage mortality on logarithm of duration of exposure (estimated from Table VI for the temperature concerned),

x = logarithm of duration (minutes) of exposure.

The mortality of a susceptible or a resistant *P. ulmi* winter egg population can be estimated in this way when the temperature and duration of the exposure are known. Mortality in a mixed population of susceptible and resistant forms can also be estimated if the proportion of each is known. When the exposure temperature is higher than −30° F it will be necessary to calculate only the mortality of the susceptible portion; if the exposure temperature is below −30° F only the mortality of the resistant form need be calculated since all susceptible form eggs will be killed.

Tests on the reliability of this method of estimating mortality have been made by comparison with observed mortality in the field and with the results of group tests in the laboratory. The field tests were not critical since no exact meteorological records for the orchard concerned were available. The comparisons indicate that estimates can be made that will be accurate within about 10 to 15%.

Estimates of mortality should be of value in apple-growing areas in predicting survival of the winter eggs and in deciding on the need for controls. They may also be useful in assigning causes of mortality where life tables are prepared, or in investigating the interaction of predators and prey. They have been of value in predicting the environmental limits of the resistant form of *P. ulmi*.

Conclusions

Populations of *P. ulmi* are capable of increasing their cold-hardiness in response to selective pressure of low winter temperatures. In areas where *P. ulmi* is not exposed to temperatures sufficiently low to cause mortality the eggs are all in the susceptible category. Where temperatures are low enough occasionally to cause some mortality some resistant individuals are found and a balanced polymorphic condition exists. Where low temperatures that kill most of the susceptible eggs occur nearly every year, as in central New Brunswick, mites are nearly all resistant.

The cold resistance of *P. ulmi* varies little due to differences in any factor so far observed other than its inheritable genetic make-up. Mortality of winter eggs increases as the duration of exposure to low temperatures in the vulnerable range increases, and the duration of exposure should be considered in estimating mortality of *P. ulmi* in the field. It is predicted that cold-hardiness of *P. ulmi* will not increase beyond the present level of the resistant form.

Acknowledgments

I wish to thank those who helped me during the progress of this work for many favors and frank suggestions. In particular Dr. F. O. Morrison of Macdonald College; Dr. R. W. Salt, Lethbridge, Alberta; Dr. J. M. Cameron, Sault Ste. Marie, Ontario; and Mr. F. T. Lord and Dr. A. D. Pickett, Kentville, Nova Scotia, who made recommendations regarding technique and equipment and other valuable suggestions and constructive criticism. The help of Mr. H. L. Crowell, Assistant Technician, Kentville, Nova Scotia, is much appreciated.

The collection of samples of *P. ulmi* outside of Nova Scotia was carried out by Mr. C. W. B. Maxwell, Fredericton, New Brunswick; Mr. B. Parent and Dr. E. J. LeRoux, St. Jean, Quebec; Mr. W. S. Babbitt, St. Thomas, Ontario; Mr. G. G. Dustan, Vineland Station, Ontario; and Dr. D. W. Clancy, Kearneysville, West Virginia, U.S.A., whose help made it possible to test samples from a much wider area than could otherwise have been done.

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OVARIOLE NUMBERS IN COLEOPTERA[1]

J. G. ROBERTSON

Abstract

A list of ovariole numbers in 329 species in 45 families of Coleoptera is presented of which about one-third are determinations made by the author. The polytrophic Adephaga have a range of 4–40 ovarioles, the acrotrophic Polyphaga, 1–200 ovarioles. The Coprinae (Scarabaeidae) are the only group containing a single ovary. A few families such as the Curculionidae contain nearly a uniform number of ovarioles. The work is briefly discussed from the viewpoint of taxonomy, evolution, and fecundity. It is suggested that ovariole numbers may be of aid to taxonomists; that multiplication, reduction, and stabilization of ovariole numbers have occurred in the evolution of beetles; and that extremes in ovariole number may reflect extremes in the level of egg production.

Introduction

In a cytotaxonomic study of the Cucujidae (Coleoptera), Robertson (31) noted a variation in ovariole number between some of the species. As ovariole number is an easily determined attribute it was decided to assess its value in a variety of species. During the course of this work a considerable but widely scattered literature on ovariole numbers in beetles was uncovered. The collective results comprise 329 species in 45 families, one-third of which have been determined by the author. Literature prior to 1847 has not been consulted but Stein's (37) monograph of that year appears to cover earlier findings rather carefully. The species and their ovariole number are listed according to Leng's *Catalogue of the Coleoptera of America, North of Mexico* (1, 2, 23, 24, 25). Certain European and South American species are assigned positions in that classification.

The discovery of Heymons' (17, 18) and Williams' (42) findings of a single ovary with a single ovariole in the Coprinae (Scarabaeidae) negates my earlier statement (32) that in insects this attribute pertains to only certain aphids.

Materials and Methods

Most of the species examined by the author were collected in the vicinity of Ottawa, Ontario. The ovaries were removed from live material after a dorsal incision from the anal pore to the thorax. In minute species like the flea beetles and certain Staphylinidae a more general dissection was necessary. The ovaries were placed on depression slides containing 0.8% saline, and the ovarioles, which are held together by connective tissue and tracheae, were separated and counted. In the list of species, the scientific or specific names in parentheses indicate the nomenclature of the author cited, and the four names in quotations are species that could not be found in catalogues of beetle taxonomy.

[1]Manuscript received February 4, 1961.
Contribution from Entomology Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario.

Results and Discussion

A specific listing and averages of ovariole numbers by families and superfamilies in beetles are shown in Tables I, II, respectively. The ovariole numbers can be separated into two groups as the ovarioles in the suborders Adephaga and Polyphaga are structurally polytrophic and acrotrophic (telotrophic), respectively.

In Adephaga the ovaries are bilateral and each ovary may contain from 4 to 40 ovarioles. In the Caraboidea, the Carabidae average 10, and have a range of 4–18, ovarioles (Table II); the Dytiscidae, however, have corresponding values of 25 and 14–40. At present the Cicindelidae and Noteridae, which average 12 and 6 ovarioles, respectively, cannot be distinguished from each other or the Carabidae. Within families an interspecific difference in ovariole number occurs in many species (Table I). The small variation in ovariole number found in some species shows that an interspecific difference of one to three ovarioles may not be significant in species represented by a single individual.

In Polyphaga the ovaries are bilateral except in the Coprinae, which contain a single ovary with one ovariole. The ovariole number is much more variable than in the Adephaga as the range is 1 to 200 ovarioles. Among superfamilies there are different degrees of overlap in ovariole number (Table II). Thus the variation in ovariole number (ranges in parentheses) is much greater in the Cantharoidea (7–70), Mordelloidea (7–200), Elateroidea (4–60), and Cucujoidea (2–61) than it is in the Staphylinoidea (4–20), Scarabaeoidea (1–7), Cerambycoidea (6–36), Curculionoidea (1–2), and Scolytoidea (2–2). A remarkable uniformity in ovariole number is seen in the Staphylinoidea, Scarabaeoidea, Curculionoidea, and Scolytoidea (Table I). In the Scarabaeoidea the value six predominates (excepting the Coprinae) as it does, but to a lesser degree, in the Staphylinoidea. In the Curculionoidea and Scolytoidea the ovariole number, with one exception, is two.

Within superfamilies, the families and subfamilies generally show the same magnitude of variation that exists between superfamilies. Consequently a clear distinction between families and subfamilies can presently be observed in only the following cases. In Cucujoidea, the Cucujidae average 3, the Coccinellidae, 17 ovarioles. In the Coccinellidae, the Coccinellinae and Epilachninae average 16 and 27 ovarioles, respectively; within Coccinellinae, the Hyperaspini, Scymini, Psylloborini, and Coccinellini have values of 7, 6, 13, and 21, in that order. In the Scarabaeidae, the Coprinae have a single ovary with one ovariole and the remaining subfamilies (Table I) have two ovaries with, usually, six ovarioles.

Table I shows that intergeneric and interspecific differences in ovariole number are frequent. An intraspecific range of three to four ovarioles occurs commonly, as is the case in certain *Coccinella*, *Crioceris*, *Galerucella*, and *Calligrapha*. However, a range as great as 10–20 ovarioles is apparent in a few species. The range of 19–70 ovarioles found in *Chauliognathus pennsylvanicus* is due to geographic differences (32). Deegener's (9) report of 300 ovarioles in *Anatis* (*Coccinella*) *ocellata* is probably a typographical error as both Gross (13) and Dobzhansky (10) found this species to have 27–30 ovarioles.

TABLE I

Ovariole number (range in parentheses) per ovary in Coleoptera

	Number	Individuals	Authority
I. ADEPHAGA: 1. CARABOIDEA			
Cicindelidae			
Cicindelini			
Cicindela punctulata Oliv.	10	1	Author
sexguttata sexguttata F.	9 (9–10)	3	"
campestris L.	— (10–12)	—	Stein (37)
Carabidae			
CARABINAE			
Carabini			
Carabus granulatus L.	— (10–12)	—	"
hortensis L.	— (10–12)	—	"
Notiophilini			
Notiophilus aquaticus L.	6	—	"
Scaratini			
Scarites subterraneus F.	6	—	Williams (41)
Clivina fossor L. (*C. arenaria* F.)	6	—	Stein (37)
HARPALINAE-BISETOSAE			
Bembidiini			
Bembidion oblongulum Mann.	7	1	Author
femoratum Sturm	8	—	Stein (37)
Pterostichini			
Pterostichus lucublandus Say	13 (13–15)	3	Author
adstrictus Eschz.	10	1	"
corvinus Dej.	11	1	"
mutus Say	15 (14–17)	2	"
melanarius Ill.	13 (11–15)	2	"
melanarius Ill. (*P. vulgaris* L.)	5	—	Gross (13)
vernalis Panz.	7	—	Stein (37)
Amarini			
Amara apricaria Payk.	7 (7–7)	3	Author
sp. A	13	1	"
sp. B	13	1	"
Agonini			
Calathus gregarius Dej.	9	1	"
Agonum bogemanni Gyll.	7 (7–7)	2	"
mulleri Hbst.	14 (13–18)	4	"
(*melanarium* group)	13 (13–13)	5	"
placidum Say	14	1	"
Lebiini			
Lebia solea Hentz	10 (10–10)	2	"
Metabletus (*Dromius*) *truncatellus* L.	4	—	Stein (37)
HARPALINAE-UNISETOSAE			
Chlaeniini			
Chlaenius tricolor Dej.	21	1	Author
sericeus Forst.	21	1	"
Harpalini			
Harpalus erraticus Say	4 (4–4)	3	"
pennsylvanicus DeG.	10 (9–13)	17	"
affinis Schrank	5 (4–6)	7	"
affinis Schrank (*H. aeneus* F.)	5	—	Gross (13)
confusus Dej.	5	—	"
sp.	7	1	Author
Anisodactylus sp.	7	1	"
Agonoderus comma F.	4 (4–4)	2	"

TABLE I—*Continued*

	Number	Individuals	Authority
	Noteridae		
Noterus crassicornis Müll.	6	—	Stein (37)
	Dytiscidae		
HYDROPORINAE			
Hydroporini			
Hydroporus palustris L.	— (14–16)	—	Stein (37)
Hyphydrini			
Hyphydrus ovatus L.	— (14–16)	—	"
COLYMBETINAE			
Agabini			
Agabus seriatus Say	20	1	Author
Colymbetini			
Colymbetes fuscus L.	— (30–40)	—	Stein (37)
DYTISCINAE			
Dytiscini			
Dytiscus marginalis L.	30	—	Henneguy (16)
HYDATICINAE			
Thermonectini			
Acilius sulcatus L.	— (30–40)	—	Stein (37)
	2. GYRINOIDEA		
	Gyrinidae		
Dineutes discolor Aubé	25	1	Author
	II. POLYPHAGA: 1. HYDROPHILOIDEA		
	Hydrophilidae		
HELOPHORINAE			
Helophorus aquaticus L.	12	1	Author
HYDROPHILINAE			
Hydrophilini			
Hydrophilus obtusatus Say	20	1	"
Hydrobiini			
Hydrobius fuscipes L.	13	—	Stein (37)
SPHAERIDIINAE			
Cercyon praetextatus Say	16 (15–16)	5	Author
	2. SILPHOIDEA		
	Silphidae		
Silphini			
Necrophorus orbicollis Say	12	1	"
Silpha surinamensis F.	22	1	"
obscura L.	12	—	Stein (37)
sinuatus F. (section *Thanatophilus*)	12	—	"
sp. (section *Necrodes*)	40	—	"

TABLE I—*Continued*

	Number	Individuals	Authority
	Scydmaenidae		
Scydmaenus tarsatus Müll.	4	—	Stein (37)
	3. STAPHYLINOIDEA		
	Staphylinidae		
OMALIINAE			
Phloeonomus lapponicus Zett.	5 (5–6)	2	Author
OXYTELINAE			
Oxytelus rugosus F.	10	—	Stein (37)
STENINAE			
Stenini			
Stenus juno F. (*S. similis* Hbst.)	6	—	"
oculatus Grav.	9	—	"
Dianous coerulescens Gyll.	5	—	"
PAEDERINAE			
Paederini			
Paederus riparius L.	6	—	Stein (37)
STAPHYLININAE			
Xantholinini			
Nudobius cephalus Say	6 (5–6)	2	Author
Xantholinus punctulatus Payk.	6	—	Stein (37)
Staphylinini			
Philonthus varius Gyll.	6	—	"
Staphylinus badipes Lec.	6	1	Author
olens Müll.	3	—	Henneguy (16)
erythropterus L.	6	—	Stein (37)
TACHYPORINAE			
Tachyporini			
Tachyporus chrysomelinus L.	6	—	"
Bolitobiini			
Bolitobius atricapillus F. (*Lordithon lunulatus* L.)	6	—	"
ALEOCHARINAE			
Myrmedoniini			
Atheta aterrima Grav. (*Homalota aterrima*)	6	—	"
"*Myrmedonia canaliculata*" (*Astilbus canaliculatus* F. ?)	7	—	"
Aleocharini			
Aleochara lanuginosa Grav.	20	—	"
	Histeridae		
HISTRINAE			
Hister sinuatus Ill.	4	—	"
Platysoma frontale Payk.	4	—	"

TABLE I—*Continued*

	Number	Individuals	Authority
4. CANTHAROIDEA			
Lampyridae			
Lucidotini			
Lucidota nigricans Say	8 (7–8)	2	Author
Photinini			
Pyractomena borealis Rand.	25	1	"
Photurini			
Photuris pennsylvanicus DeG.	27	1	"
Cantharidae			
Chauliognathini			
Chauliognathus pennsylvanicus DeG.	44 (19–70)	25	Author
Cantharini			
Cantharis bilineatis Say	25	1	"
rufa L.	52	1	"
Melyridae			
MALACHIINAE			
Collops tricolor Say	13	1	Author
Malachius aeneus L.	56	1	"
Anthocomus fasciatus L. (*Malachius fasciatus*)	9	—	Stein (37)
Cleridae			
CLERINAE			
Clerini			
Thanasimus (*Clerus*) *formicarius* L.	12	—	Stein (37)
Trichodes nutalli Kby.	17	1	Author
5. MORDELLOIDEA			
Oedemeridae			
OEDEMERINAE			
Oedemerini			
Oedemera virescens L.	7	—	Stein (37)
Meloidae			
Mylabrini			
Mylabris geminata F.	12	—	Henneguy (16)
Lyttini			
Epicauta pennsylvanica DeG.	50	1	Author
Lytta vesicatoria L.	80	—	Henneguy (16)
Meloini			
Meloe sp.	200+	—	Imms (20)
Anthicidae			
Notoxus monoceros L.	8	—	Stein (37)
6. ELATEROIDEA			
Elateridae			
"Elaterids"	4	—	Henneguy (16)

TABLE I—*Continued*

	Number	Individuals	Authority
PYROPHORINAE			
Pyrophorini			
Alaus oculatus L.	4	—	Williams (41)
Conoderus lividus DeG.	4	—	"
Lacon murinus L. (*Elater murinus*)	— (30–36)	—	Stein (37)
Lepturoidini			
Athous acanthus Say	16	2	Author
Ctenicera (*Ludius*) *holosericeas* Oliv. (*Prosternon tessallata* L.)	8	—	Stein (37)
aena L. (*Elater aeneus*)	— (30–36)	—	"
(*Colymbites aeneus* L.)	55 (50–60)	—	Horst (19)
aeripennis destructor Brown	56 (56–56)	2	Zacharuk (42)
ELATERINAE			
Agriotini			
Agriotes (*Elater*) *aterrimus* L.	— (25–30)	—	Stein (37)
obscurus L.	40 (36–48)	—	Horst (19)
Elaterini			
Ampedus (*Elater*) *sanguineus* L.	35 (30–40)	—	"
rubricus Say	5	1	Author
Melanotini			
Melanotus (*fissilis* group)	37 (36–37)	3	"
Buprestidae			
Buprestini			
Dicerca divaricata Say	12 (12–12)	2	"
Melanophila acuminata DeG.	10	—	Williams (41)
7. Dryopoidea			
Elmidae			
Elmini			
Elmis maugei megerlei Duft. (*E. aeneus* Müll.)	4	—	Stein (37)
Macronychus glabratus Say	10 (10–10)	2	Author
Heteroceridae			
Heterocerus brunneus Mels.	6 (6–6)	2	Author
8. Dascilloidea			
Dermestidae			
DERMESTINAE			
Dermestini			
Dermestes lardarius L.	9	—	Stein (37)
Attagenini			
Attagenus piceus Oliv.	4	1	Author
Byrrhidae			
BYRRHINAE			
Byrrhini			
Byrrhus pilula L.	18	—	Stein (37)

TABLE I—*Continued*

	Number	Individuals	Authority
	9. CUCUJOIDEA		
	Nitidulidae		
NITIDULINAE			
Nitidulini			
Epurea (*Nitidula*) *obsoleta* F.	9	—	Stein (37)
	Cucujidae		
Silvanini			
Oryzaephilus surinamensis L.	3 (3–3)	5	Author
mercator Fauv.	3 (3–3)	5	"
Silvanus bidentatus F.	3	1	"
gemellatus Duv.	3 (3–3)	3	"
Ahasverus advena Waltl.	3 (3–3)	3	"
Cryptolestes turcicus Grouv.	2 (2–2)	3	"
ugandae Steel and Howe	2 (2–2)	10	"
pusilloides Steel and Howe	2 (2–2)	10	Robertson (31)
pusillus Schönh.	3 (3–3)	34	Author
spartii Curtis	3 (3–3)	10	Robertson (31)
ferrugineus Steph.	4 (4–4)	5	Author
Laemophloeus monilis F.	8	—	Stein (37)
	Erotylidae		
Dacnini			
Dacne (*Engis*) *bipustulata humeralis* F.	5	—	Stein (37)
	Mycetophagidae		
Mycetophagini			
Typhea stercorea L.	7 (6–7)	5	Author
Triphyllus bifasciatus F. (*Litargus connexus bifasciatus* Geoffr.)	4	—	Stein (37)
	Lathridiidae		
Lathridiini			
Lathridius porcatus Hbst.	3	—	Stein (37)
	Phalacridae		
Stilbus apicalis Melsh.	8 (7–8)	5	Author
	Coccinellidae		
COCCINELLINAE			
Hyperaspini			
Hyperaspis undulata Say	6 (6–6)	3	"
Brachyacantha ursina F.	7	1	"
Scymnini			
Stethorus punctillum Wse.	2	—	Dobzhansky (10)
Nephus redtenbacheri Muls.	6	—	"
Pullus ater Kug.	4	—	"
suturalis Thunb.	7	—	"
subvillosus Goeze	7	—	"
haemorrhoidalis Wse.	6	—	"
Scymmus interruptus Goeze	6	—	"
rubromaculatus Goeze	6	—	"
nigrinus Kug.	6	—	"
frontalis F.	6	—	"
Coccidulini			
Coccidula pectoralis F. (*C. rufa* Hbst.)	7	—	Stein (37)
Psylloborini			
Psyllobora viginti-maculata Say	10	1	Author

TABLE I—*Continued*

	Number	Individuals	Authority
Coleomegilla maculata lengi Timb.	19	1	Author
Thea 22-punctata L.	— (11–12)	—	Dobzhansky (10)
Vibidia 12-guttata Poda	12	—	"
Halyzia 16-guttata L.	14	—	"
Coccinellini			
Anisosticta 19-punctata L.	12	—	"
Adonia variegatata Goeze	— (22–25)	—	"
Hippodamia tredecimpunctata tredecimpunctata L.	— (24–30)	—	"
tredecimpunctata tibialis Say	22	1	Author
parenthesis Say	13 (11–15)	3	"
7-maculata DeG.	— (20–23)	—	Dobzhansky (10)
convergens Guer.	12	—	Williams (41)
Coccinella redimita Wse.	10	—	Dobzhansky (10)
14-pustulata L.	10	—	"
hieroglypha L.	14	—	"
5-punctata L.	— (10–18)	—	"
	— (14–16)	—	Stein (37)
trifasciata perplexa Muls.	16 (15–17)	4	Author
septempunctata L.	30	—	Stein (37)
	— (41–61)	—	Dobzhansky (10)
11-punctata L.	— (32–36)	—	Dobzhansky (10)
novemnotata novemnotata Hbst.	31 (30–32)	6	Author
divaricata Oliv.	— (33–37)	—	Dobzhansky (10)
transversoguttata quinquenotata Kby.	43	2	Author
Adalia 10-punctata L.	10	—	Dobzhansky (10)
bipunctata L.	24 (24–24)	4	Author
Semiadalia 11-notata Schn.	— (21–36)	—	Dobzhansky (10)
Harmonia 4-punctata Pont.	— (18–20)	—	"
axyridis Pall.	— (27–39)	—	"
Anisocalvia (Calvia) 10-guttata L.	— (14–15)	—	"
14-guttata L.	— (20–29)	—	"
Anatis ocellata L.	— (27–29)	—	"
(Coccinella) ocellata L.	30	—	Gross (13)
quindecimpunctata Oliv.	22 (22–22)	2	Author
Neomysia (Paramysia) oblongoguttata L.	— (22–24)	—	Dobzhansky (10)
Myrrha 18-guttata L.	8	—	"
Tytthaspis 16-guttata L.	— (10–11)	—	"
Synharmonia lyncea Oliv.	10	—	"
conglobata L.	12	—	"
Chilocorini			
"Chilocorus quadripustulatus"	10	—	Stein (37)
EPILACHNINAE			
Subcoccinella vigintiquatorpunctata L. (*Coccinella globosa* Schn.)	16	—	Stein (37)
Subcoccinella vigintiquatorpunctata L. (*Coccinella globosa* Schn.)	— (16–18)	—	Dobzhansky (10)
Epilachna pusillanima Muls.	— (30–33)	—	"
11-variolata Boisd.	— (41–43)	—	"
28-maculata Motsch.	— (27–28)	—	"
10. TENEBRIONOIDEA			
Alleculidae			
ALLECULINAE			
Allecula morio F.	— (30–40)	—	Stein (37)
Isomira sp.	19	1	Author

TABLE I—*Continued*

	Number	Individuals	Authority
TENEBRIONINAE			
Tenebrionidae			
Blaps sp.	30	—	Henneguy (16)
Tenebrio molitor L.	12	—	Schlottman and Bonhag (33)
DIAPERINAE			
Diaperis maculata Oliv.	8 (7–8)	8	Author
boleti L.	10	—	Stein (37)
ULOMINAE			
Tribolium castaneum Hbst.	4	—	Srivastava (36)
HELOPINAE			
Helops caraboides Panz. (*Cylindronotus laevioctostriatus* Goeze)	52	—	Stein (37)
Lagriidae			
Lagria hirta L.	— (30–40)	—	Stein (37)
Arthromacra aena Say	30	1	Author
11. BOSTRICHOIDEA			
Anobiidae			
ANOBIINAE			
Dryophilini			
Ernobius mollis L.	12	—	Gardiner (11)
Anobiini			
Anobium punctatum DeG. (*A. pertinax* L.)	6	—	Stein (37)
Ptilinini			
Ptilinus pectinicornis L.	16	—	Gardiner (12)
Bostrichidae			
Bostrichus sp.	2	—	Stein (37)
Lyctidae			
Lyctus brunneus Steph.	12	—	Parkin (29)
Cisidae			
CISINAE			
Cis boleti Scop.	6	—	Stein (37)
12. SCARABAEOIDEA			
Scarabaeidae			
COPRINAE			
Scarabaeini			
Scarabaeus sacer L.	1	—	Heymons (18)
semipunctatus F.	1	—	" (17)
variolosus F.	1	—	" "
laticollis L.	1	—	" "

TABLE I—*Continued*

	Number	Individuals	Authority
Sisyphus schaefferi L.	1	—	Heymons (17)
Canthon rutilans Casteln. var. *cyanescens* Dej.	1	—	" "
Coprini			
Pinotus carolinus L.	1	—	Williams (41)
anaglypticus Mannerh.	1	—	Heymons (17)
Copris hispanus L.	1	—	"
APHODIINAE			
Aphodiini			
Aphodius fimetarius L.	7	—	Stein (37)
GEOTRUPINAE			
Bolbocerini			
Bolbocerosoma farctum F.	1	—	Williams (41)
Eucanthus lazarus F.	1 (1–2)	—	"
Geotrupini			
Geotrupes stercorarius L.	6	—	Stein (37)
sylvaticus Panz.	6	—	Gross (13)
TROGINAE			
Trox sabulosus	5	—	Stein (37)
MELOLONTHINAE			
Sericini			
"*Omaloplia brunnea*" (*Serica brunnea* L. ?)	6	—	Stein (37)
Melolonthini			
Diplotaxis tristis Kirby	6	5	Author
Melolontha vulgaris L.	6	—	Stein (37)
Phyllophaga fusca Froel.	6 (6–6)	5	Author
futilis LeC.	6 (6–6)	5	"
sp.	6	—	Williams (41)
Phyllopertha horticola L.	6	—	Stein (37)
	6	—	Milne (28)
Macrodactylini			
Dichelonyx subvittata LeC.	6 (6–6)	4	Author
Macrodactylus subspinosus F.	6	—	Bryan (3)
Hopliini			
Hoplia trifasciata Say	5	1	Author
RUTELINAE			
Anomalini			
Popillia japonica Newn.	5 (5–6)	2	Author
	6	—	Williams (41)
DYNASTINAE			
Oryctini			
Bothynus relictus Say	6 (5–6)	2	Author
(*Ligyrus*) *gibbosus* DeG.	6	—	Williams (41)
Dynastini			
Dynastes tityus L.	6	—	"
CETONIINAE			
Gymnetini			
Cotinis nitida L.	6	—	"
Cetoniini			
Cetonia aurata L.	6	—	Gross (13)
Trichiini			
Trichius fasciata L.	6	—	Gross (13)

TABLE I—*Continued*

	Number	Individuals	Authority
	Lucanidae		
DORCINAE			
Dorcus (*Lucanus*) *parallelipipedus* L.	12	—	Stein (37)
	Passalidae		
PSEUDACANTHINAE			
Popilius disjunctus Ill. (*Passalus cornutus* F.)	2	—	Bryan (3)
	2	—	Krause (22)
	2	—	Williams (41)
	13. CERAMBYCOIDEA		
	Cerambycidae		
CERAMBYCINAE			
Cerambycini			
Cerambyx cerdo heros Scop.	— (30–36)	—	Stein (37)
Lepturini			
Gaurotes abdominalis Bland	11	1	Author
Clytini			
Megacyllene robiniae Forst.	27	1	"
LAMIINAE			
Monochamini			
Monochamus scutellatus Say	11	1	"
Acanthoderini			
Aegomorphus decipiens Hald.	20	2	"
Tetraopini			
Tetraopes tetrophthalmus Forst.	30	6	"
	Chrysomelidae		
CRIOCERINAE			
Crioceris asparagi L.	10 (8–13)	15	"
duodecimpunctata L.	11 (11–12)	5	"
Lema trilineata Oliv.	6	1	"
CLYTRINAE			
Clytrini			
Anomoea laticlavia laticlavia Forst.	16	2	Author
CHLAMYDINAE			
Chlamisus chamaedaphnes Brown	4 (4–5)	8	"
eubati Brown	3 (3–4)	2	"
CRYPTOCEPHALINAE			
Cryptocephalini			
Cryptocephalus quadruplex Newn.	7	1	"
Bassareus sellatus Suffr.	8 (8–8)	3	"
EUMOLPINAE			
Typophorini			
Paria sp.	7	2	"
Corynodini			
Chrysochus auratus F.	15	6	"

TABLE I—*Continued*

	Number	Individuals	Authority
CHRYSOMELINAE			
Timarchini			
Timarcha coriaria Laich.	5	—	Gross (13)
Chrysomelini			
Labidomera clivicollis Kirby	21 (19–21)	3	Author
Leptinotarsa decemlineata Say	25 (25–25)	2	"
Chrysolina (*Chrysomela*) *hyperica* Forst.	10	—	Deegener (9)
(*Chrysomela*) *sanguinolenta* L.	16	—	Stein (37)
Chrysomela (*Lina*) *populi* L.	20	—	Gross (13)
mainensis mainensis Bech.	16	1	Author
Calligrapha multipunctata bigsbyana Kby.	17 (15–18)	10	"
philadelphica L.	16 (14–18)	13	"
vicina Schffr.	16 (14–17)	10	"
rowena Knab	16 (15–17)	2	"
alni Schffr.	13 (12–14)	2	"
confluens Schffr.	22 (21–23)	3	Author
apicalis Notm.	17 (16–18)	3	"
alnicola Brown	17 (16–18)	6	"
pnirsa Stal.	22 (21–23)	4	"
amator Brown	22	1	"
virginea Brown	17 (16–18)	6	"
Phaedonini			
Plagiodera versicolora Laich.	15 (15–17)	8	"
Phyllodectini			
Gonioctena (*Phytodecta*) *olivacea* Forst.	10 (8–12)	40	Woloff and Richards (40)
fornicata Brügg	18	—	Voukassovitch (39)
GALERUCINAE			
Atysini			
Trirhabda virgata LeC.	15	2	Author
borealis Blake	11	1	"
canadensis Kirby	15	5	"
Galerucella xanthomelaena Schrank	27 (25–28)	16	"
alni Fall	6	1	"
HALTICINAE			
Disonychini			
Disonycha triangularis Say	13	1	"
Crepidoderini			
Epitrix sp.	6	—	Williams (41)
Aphthonini			
Longitarsus sp.	7	1	Author
CASSIDINAE			
Cassida viridis L. (*C. equestris* F.)	7	—	Stein (37)
14. CURCULIONOIDEA			
Platystomidae			
Anthribini			
Anthribus sp.	2	—	Stein (37)
Curculionidae			
RHYNCHITINAE			
Rhynchitini			
Rhynchites sp.	2	—	Stein (37)

TABLE I—*Continued*

	Number	Individuals	Authority
APIONINAE			
Apion pomonae F.	2	—	Stein (37)
OTIORHYNCHINAE			
Polydrusini			
Polydrosus sp.	2	—	"
mollis Ström	2	—	Leńkowa (26)
sericeus Schall	2	—	"
pilosus Gredler	2	—	"
Sciaphilini			
Sciaphilus asperatus Bonsd.	2	1	Author
Cneorhinini			
Cneorhinus sp.	2	—	Stein (37)
Brachyderini			
Brachyderes (*Thylacites*) sp.	2	—	"
Brachyrhinini			
Brachyrhinus (*Otiorhynchus*) sp.	2	—	"
sulcatus F.	2	—	Cram (7)
ovatus L.	2 (2–2)	4	Author
Phyllobiini			
Phyllobius sp.	2	—	Stein (37)
CURCULIONINAE			
Sitonini			
Sitona hispidulus F.	2 (2–2)	3	Author
lepidus Gyll.	2 (2–2)	3	"
scissifrons Say	2 (2–2)	2	"
Hyperini			
Hypera punctata F.	2 (2–2)	3	"
nigrirostris F.	2 (2–2)	2	"
(*Phytonomus*) sp.	2	—	Stein (37)
Hylobiini			
Hylobius (*Hypera*) *abietis* L.	2	—	Gross (13)
Hylobius sp.	2	—	Stein (37)
Erirhinini			
Dorytomus sp.	2	—	"
Balanini			
Curculio (*Balanimus*) sp.	2	—	"
Cionini			
Cionus sp.	2	—	"
Anthonomini			
Anthonomus pomorum L.	2	—	Metcalfe (27)
grandis Boheman	2	—	Burke (4)
Mecinini			
Gymnetron antirrhini Payk.	2 (2–2)	3	Author
Cleonini			
Cleonus sp.	2	—	Stein (37)
Lixus sp.	2	—	"
COSSINAE			
Cossonini			
Cossonus linearis F.	2	—	"
Pentarthrum huttoni Woll.	2	—	Chararas (5)
Caulotrupis aeneopiceus Boh.	2	—	"
Rhyncolini			
Eremotes (*Rhyncolus*) *elongatus* Gyll.	2	—	"
porcatus Germ.	1	—	"

TABLE I—*Concluded*

	Number	Individuals	Authority
CALENDRINAE			
Calendrini			Chararas (5)
Sitophilus granarius L.	2	—	"
oryzae L.	2	—	Khan (21)
Calendra parvula Gyll.	2 (2–2)	2	Author
15. SCOLYTOIDEA			
Scolytidae			
SCOLYTINAE			
Scolytus sp.	2	—	Henneguy (16)
scolytus F.	2	—	Chararas (5)
HYLESININAE			
Hylesinini			
Leperisinus (*Hylesinus*) *fraxini* Panzer	2	—	"
Blastophagus piniperda L. (*Hylesinus piniperda*)	2	—	Stein (37)
Hylastes sp.	2 (2–2)	2	Author
Dendroctonus monticolae Hopk.	2	—	Reid (30)
Phloeosinus bicolor Brullé	2	—	Chararas (5)
canadensis Sw.	2 (2–2)	2	Author
IPINAE			
Ipini			
Ips typographus L.	2	—	Chararas (5)
perturbatus Eich.	2	1	Author
Pityogenes chalcographus L.	2	—	Chararas (5)
hopkinsi Sw.	2	1	Author
Orthotomicus vicinus LeC.	2	—	"
Xylechinus pilosus Ratz.	2	—	Stein (37)

TABLE II

Averages of ovariole numbers by families and superfamilies in Coleoptera

Group	No.	Range	Species	Genera
Caraboidea	12	4–40	41	23
Cicindelidae	10	9–12	3	1
Carabidae	10	4–21	31	15
Noteridae	6	—	1	1
Dytiscidae	25	14–40	6	6
Gyrinoidea	25	—	1	1
Gyrinidae	25	—	1	7
Hydrophiloidea	15	12–20	4	4
Hydrophilidae	15	12–20	4	4
Silphoidea	17	4–40	6	3
Silphidae	20	12–40	5	2
Scydmaenidae	4	—	1	1

TABLE II—*Concluded*

Group	No.	Range	Species	Genera
Staphylinoidea	7	3–20	19	16
Staphylinidae	7	3–20	17	14
Histeridae	4	4–4	2	2
Cantharoidea	26	7–70	11	11
Lampyridae	20	7–27	3	3
Cantharidae	40	19–70	3	2
Melyridae	26	9–56	3	3
Cleridae	14	12–17	2	2
Mordelloidea	60	7–200	6	6
Oedemeridae	7	—	1	1
Meloidae	86	12–200	4	4
Anthicidae	8	—	1	1
Elateroidea	21	4–60	15	11
Elateridae	22	4–60	13	9
Buprestidae	11	10–12	2	2
Dryopoidea	7	4–10	3	3
Elmidae	7	4–10	2	2
Heteroceridae	6	6–6	1	1
Dascilloidea	10	4–18	3	3
Dermestidae	7	4–9	2	2
Byrridae	18	—	1	1
Cucujoidea	14	2–61	72	39
Nitidulidae	9	—	1	1
Cucujidae	3	2–8	12	5
Erotylidae	5	—	1	1
Mycetophagidae	6	4–7	2	2
Lathridiidae	3	—	1	1
Phalacridae	8	7–8	1	1
Coccinellidae	17	2–61	54	28
Tenebrionoidea	21	4–52	10	9
Alleculidae	27	19–40	2	2
Tenebrionidae	20	4–52	6	5
Lagriidae	33	30–40	2	2
Bostrichoidea	9	2–16	6	6
Anobiidae	11	6–16	3	3
Bostrichidae	2	—	1	1
Lyctidae	12	—	1	1
Cisidae	6	—	1	1
Scarabaeoidea	4	1–12	34	26
Scarabaeidae	4	1–7	32	26
Lucanidae	12	—	1	1
Passalidae	2	2–2	1	1
Cerambycoidea	15	6–36	46	28
Cerambycidae	22	11–36	6	6
Chrysomelidae	13	6–28	40	22
Curculionoidea	2	1–2	38	25
Platystomidae	2	—	1	1
Curculionidae	2	1–2	37	24
Scolytoidea	2	2–2	14	10
Scolytidae	2	2–2	14	10

The common taxonomic grouping of the Curculionoidea and Scolytoidea under Rhynchophora is well supported by the ovariole number as both groups contain two ovarioles. The frequent occurrence of six ovarioles in the Scarabaeidae and Staphylinidae, groups that are widely separated by Leng (23), may be further evidence for their close relationship recognized by Crowson (8). The variation of 4–40 ovarioles in the Silphoidea sharply separates this group from the Staphylinoidea, which includes the Silphoidea in the arrangement of Crowson. Among most groups and species, however, abrupt changes in the ovariole number make an interpretation in terms of natural classification difficult.

It is interesting to analyze some evolutionary aspects of ovariole number. Ovarioles, of course, are a product of morphogenesis. Germ cells differentiate in cleavage and formative processes carry them to the dorsal wall of the coelomic sacs. Snodgrass (34, 35) states that with the disappearance of the intercoelomic septa, the splanchnic mesoderm containing the germ cells forms a continuous genital ridge which then segments to form the ovarioles. He regards the genital ridge as the primordial gonad in insects. Gustafson (14) believes that in some insects the coelomic sacs differentiate directly into segmental gonads. He suggests that there is evidence for an insect prototype that contained nine pairs of gonads arranged metamerically and that, numerically, ovaries evolve in the direction of two ovaries each with a single or few ovarioles. The Rhynchophora, which are widely considered the most advanced Coleoptera, have two ovarioles per ovary, a fact that may support Gustafson's concept of the direction of evolution. However, the widespread occurrence of high and low ovariole numbers in groups like the Mordelloidea, Cucujoidea, and Cerambycoidea, suggests that the expedience of evolving a high number is as great as evolving a low one. In coleopteran evolution, therefore, ovariole number multiplies and reduces freely in some groups, and is highly stabilized in others.

The relationship of ovariole number to egg production is obscure in beetles. It is tempting to suppose that the 200 ovarioles in *Meloe* sp. reflect a high fecundity which, in turn, is related to a high larval mortality resulting from their uncertainty in finding bee eggs to feed upon. The two ovarioles in Scolytidae may indicate a low fecundity that reflects their relatively sheltered existence and easy access to food. Indeed it has been reported that Meloidae produce as many as 10,000 eggs (20) and that *Dendroctonus piceaperda* Hopk. (Scolytidae) produces 80–150 eggs (38). However, many other factors such as nutrition and temperature are undoubtedly important to egg production. Furthermore, a high egg production in the laboratory may not indicate a high egg production in nature. Clark (6) showed that *Chrysomela gemellata* Rossi and *C. hyperica* Forst. oviposit nearly twice the number of eggs in their laboratory environment, arranged to simulate their natural environment, than they oviposit in nature. Accordingly, any effort directed to a study of ovariole number and egg production must take cognizance of the many factors that may influence the results.

Acknowledgments

It is a pleasure to thank Mr. W. J. Brown, Entomology Research Instit-

ute, Ottawa, Ontario, who identified most of the species and assisted in problems of synonymy. Drs. H. F. Howden and E. C. Becker of that Institute identified the species of Scarabaeidae and Elateridae, respectively; Dr. G. E. Ball, Department of Entomology, University of Alberta, Edmonton, Alberta, identified certain Carabidae. Dr. C. W. Rutschky, Department of Zoology and Entomology, Pennsylvania State University, University Park, Pa., provided laboratory facilities during a collecting period in the vicinity.

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THE SALIVARY GLANDS OF AEDES AEGYPTI

HISTOLOGICAL–HISTOCHEMICAL STUDIES[1]

C. W. M. Orr,[2] Anne Hudson,[3] and A. S. West

Abstract

The maturation of the salivary glands in adult mosquitoes was studied from histological preparations. A striking sexual dimorphism in the glands apparently reflects the blood-feeding habits of the female. The glands of the female undergo a dramatic development, following emergence, whereas those of the male remain small and show little change. Three main regions of secretory activity differentiate in the female: the necks and the acini proper of the lateral lobes and the acinus of the central lobe. These areas are characterized by histochemical criteria. A cycle of secretory activity occurs after ingestion of blood. The possible functions of the secretion products are discussed.

Introduction

The salivary glands of mosquitoes have received a great deal of attention because of their role in the spreading of disease organisms. This attention has been somewhat restricted to a description of the glands as a location of parasitic organisms and accounts of general structure and cytology are comparatively few. It is convenient to mention these anatomical and histological studies under two main headings: those which attempt to define specific characteristics, and those which are concerned with the secretions of the glands and their effect upon the vertebrate host, particularly man. Only the more pertinent literature will be mentioned here; a more complete review is in preparation (23).

In 1901, Christophers (4) reported on an anatomical and histological study of the adult female mosquito and included a description of the salivary glands. In 1960 (5) he described those of *Aedes aegypti* (L.) and compared them with the glands of some other species. Shishlyaeva-Matova[4] (24) described the morphology and histology of the salivary glands of *Anopheles sacharovi* Favr. and suggested the occurrence of specific morphological characteristics. This suggestion has been supported by the work of Samnotra (22) with 15 species of mosquitoes. Samnotra also observed that a period of about 72 hours following emergence was required before the glands appeared mature. Barth

[1]Manuscript received February 17, 1961.
Contribution from the Department of Biology, Queen's University, Kingston, Ontario, and the Queen's University Biological Station. These studies were initiated with the support of grants from the Defence Research Board of Canada (DRB 6801-19) and the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service (E-1155), and were continued in the Department of Biology, The Johns Hopkins University, with support from grant E-2358 to Dr. D. R. Evans from the National Institute of Allergy and Infectious Diseases. We are deeply indebted to Dr. Evans and to Dr. R. R. Cowden, Division of Cell Biology, Institute for Muscle Disease, New York, for helpful advice and criticism, particularly on the histochemical studies. We should also like to acknowledge the help of Dr. T. R. F. Wright, Department of Biology, The Johns Hopkins University, with photography.

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[4]Appreciation is expressed to J. R. Allen for translation of the Russian.

(2) made a detailed histological study of the glands of *Aedes scapularis* Rondani. These studies and others have together demonstrated differential staining and cellular variation of the central and lateral lobes, various relationships between the sizes of the lobes, and their division into anterior and posterior regions. Accessory lobes have also been described. The probability of a complex of secretory products has been suggested.

The secretions of the glands have been little studied *in situ*. Barth (2) described two types, a lipocrine material produced by the two lateral lobes and a rhagiocrine material by the central lobe. These terms were assigned on the basis of observations of the way in which the droplets were formed within the lobes and upon some histochemical tests. The main body of literature under this heading concerns the production of anticoagulins and agglutinins; much of this work has been summarized by Christophers (5). McKiel (15), Hudson *et al.* (10), and others have shown that the reaction to mosquito bites is of an allergic type and is produced in response to injections of saliva.

In addition to his observations on the effect of salivary gland homogenates on the coagulation of blood, Metcalf (18) attempted to demonstrate enzyme activity in the glands of *Anopheles quadrimaculatus* Say; his results were negative.

The present work is an attempt to establish the presence of a number of components by histochemical tests and describes the growth and structure of the glands of young and mature female and male *Aedes aegypti*. The work was undertaken as one part of a major study of the reactions to bites of mosquitoes and other blood sucking arthropods (26).

Materials and Methods

The mosquitoes used in these experiments were *Aedes aegypti* from a laboratory colony that has been maintained at Queen's University for a number of years. The larvae were reared on a suspension of rabbit food in rectangular enamel trays. The pupae were isolated and the resultant adults transferred to dry vials. The emergence time was noted and the adults kept without food or water until required. Ambient temperatures during the period of study ranged from 65° to 80° F, and consequently small variations were to be expected due to differences in rate of development.

The material for histological study was fixed in either alcoholic Bouin's or Carnoy's fixatives; both gave equally good results. Following fixation the mosquitoes were dehydrated in graded changes of ethanol, cleared in benzene, and embedded in Fisher's tissuemat (melting point 58–60° C). Sections were cut at 6 μ and stained by the azan trichrome method of Heidenhain.

Sections of salivary glands of unfed males and females, fixed immediately after emergence and every 12 hours thereafter up to 72 hours, were examined. The effect of the ingestion of blood upon the salivary glands was observed in females 6, 12, 24, 48, 72, 120, and 162 hours after feeding. Sections of the glands of at least three mosquitoes were examined at each stage.

Histochemical preparations were made from Carnoy-fixed, mature mosquitoes. Initially Himes's and Moriber's (8) triple stain for DNA, polysaccharides, and proteins was used as a survey stain. The PAS stain of McManus (17) was used to determine the distribution of carbohydrate grouping. Loss

of PAS staining following treatment with diastase (1% for 2 hours at 37° C) was taken to indicate the presence of glycogen. Mucopolysaccharides were identified by the PAS/alcian blue technique of Mowry (19) or by the presence of metachromasia after staining with azure A in 30% alcohol (Kramer and Windrum (12)). Acid proteins were demonstrated by the methylene blue method of Singer and Wislocki (25). Mercuric bromphenol was used for basic proteins (Mazia *et al.* (14)).

The dibasic amino acids lysine, arginine, and histidine were stained according to Deitch (6) and SH groups according to Barrnett and Seligman (1). Lillie's (13) and Glenner and Lillie's (7) methods were used for tryptophan and tyrosine respectively. Lipids were stained with Sudan black B (Pearse (21)). Staining by azure B bromide, removable by ribonuclease, was taken to represent RNA (Pearse (21)).

Results

The Mature Salivary Glands of the Female Mosquito

The salivary glands are paired trilobed structures arising close to the neck and extending posteriad on either side of the gut as far as the junction of the meso- and meta-thorax. They lie on either side of the proventriculus and just dorsal to the thoracic ganglion. Their location and appearance has frequently been described (5). Briefly, each lobe consists of an anterior neck confluent with a posterior acinus; in the lateral lobes the neck corresponds to the "anterior" or "proximal" region and the acinus to the "posterior" or "distal" regions of Christophers (5) and other authors. See Figs. 1 and 2.

The main salivary duct extends from the hypopharynx to the beginning of the neck region where it divides into two; it passes through the neck as a pair of ducts which lead to the glands of each side. Inside the glands the ducts branch again and extend almost to the full length of each of the lobes. A layer

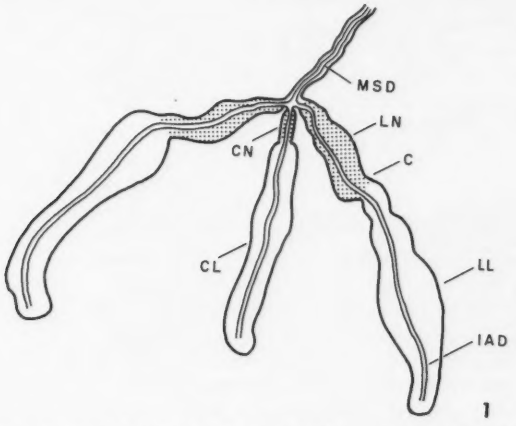


FIG. 1. Outline diagram of female salivary glands; one side, showing main regions referred to in text. MSD, main salivary duct (one branch); LN, lateral neck; C, constriction; LL, lateral lobe (acinus); IAD, intra-acinar duct; CN, central neck; CL, central lobe (acinus).

of flattened cells with oblong nuclei surround the main ducts, which are supported by strong taenidial thickenings. There are fewer taenidia within the lobes although they are still conspicuous (Fig. 3). The ducts dilate within the acini where the taenidial rings are increasingly difficult to distinguish and we found, in agreement with Bhatia *et al.* (3), that they appear to be absent in the posterior parts of the acini.

The central lobe has a short, narrow neck of uniform thickness (Figs. 2 and 4) and is composed of small cells with distinct nuclei. An abrupt dilation joins the neck and the cylindrical acinus where large, regular cells form a single layer around the duct. The small nuclei of the acinar cells lack a distinct nucleolus at this stage, and lie close to the periphery of the cell (Fig. 5).

The lateral lobes are similar to one another in structure and staining properties. They are longer, wider, and more convoluted than the central lobe and the distinction between the neck and acinus is not so sharp. The neck is much longer than that of the central lobe. It varies in width, narrowing and staining heavily at the anterior and posterior ends; medially the cells are larger and contain globules of deeply staining material which are also particularly evident just before the junction of the neck and acinus (Fig. 13). The nuclei are large, spherical, and well defined. In dissections a constriction is evident at the junction of the neck and acinus and this appears to be the region at which the staining properties of the neck and acinus differ. The morphology of the acinar cells is similar to that of the central lobe, but the lateral acinar cells are a little larger and the cytoplasm is more coarsely granular. Occasionally accessory acini may be observed (Fig. 6).

The Development of the Salivary Glands of the Female Mosquito

Immediately after emergence of the female the glands are small, thin, and unconvoluted. There are no obvious differences between the lobes and no division into a neck and acinus is apparent at this stage. Two regions of about equal size may be distinguished in each small, regular cell: an inner less densely basophilic region and an outer part containing a large intensely azanophilic nucleus surrounded by a basophilic region. Though numerous sections of the glands were examined for mitotic figures, none was observed. It is probable, therefore, that the glands grow by increase in cell volume rather than number, as in *Anopheles albimanus* Wied. (11), and in *A. scapularis* (2). However, Shishlyaeva-Matova (24) stated that mitotic figures were observed repeatedly in the cells of the constricted regions between the necks and acini of the lateral lobes in *Anopheles sacharovi* Favr. The main characteristics of the mature gland are formed during the 36 hours following emergence. The lateral lobes become convoluted and longer than the central lobe; secretion fills the greater part of the acinar cells and the nuclei are more peripherally placed (Fig. 7). By 72 hours the nuclear size has decreased and the cytoplasmic volume has increased, and by the end of the third day the glands are mature; there are no further significant morphological changes. It should be noted that whilst our observations are based on 72-hour-old females, the time taken by the glands to reach maturity may in fact be less than this. It is to be expected that the rate of development is in part dependent upon the predominant temperature at and following emergence.

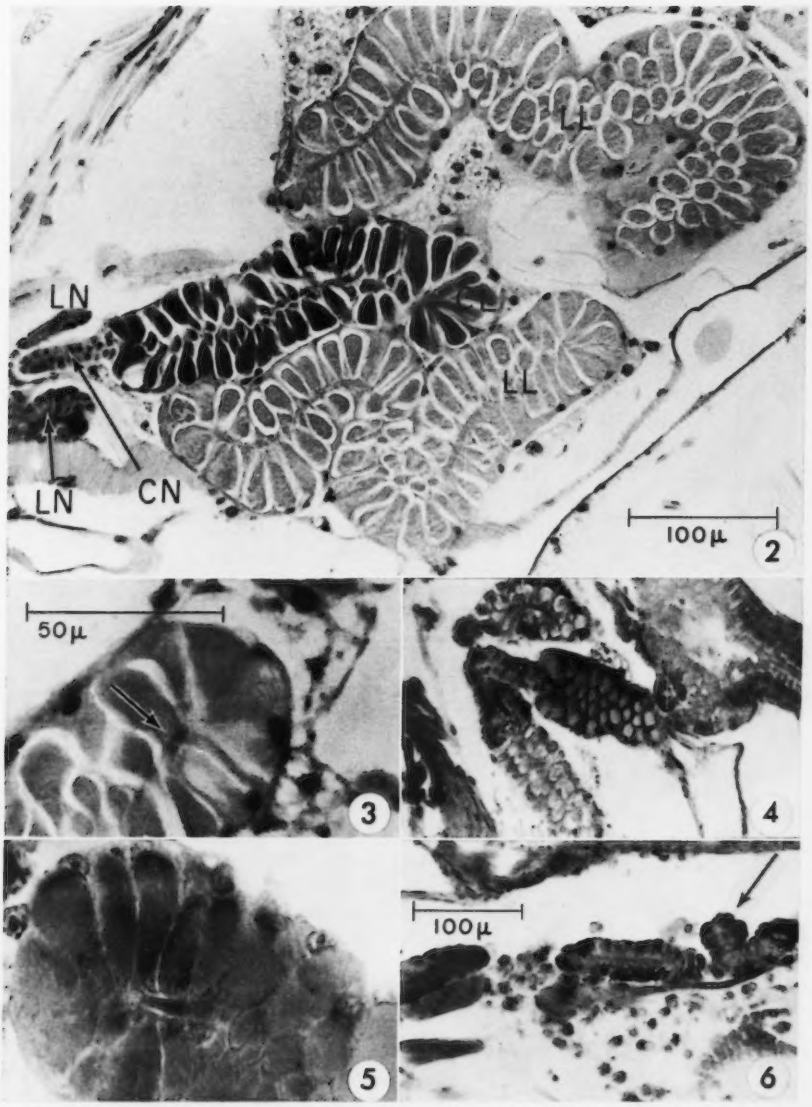


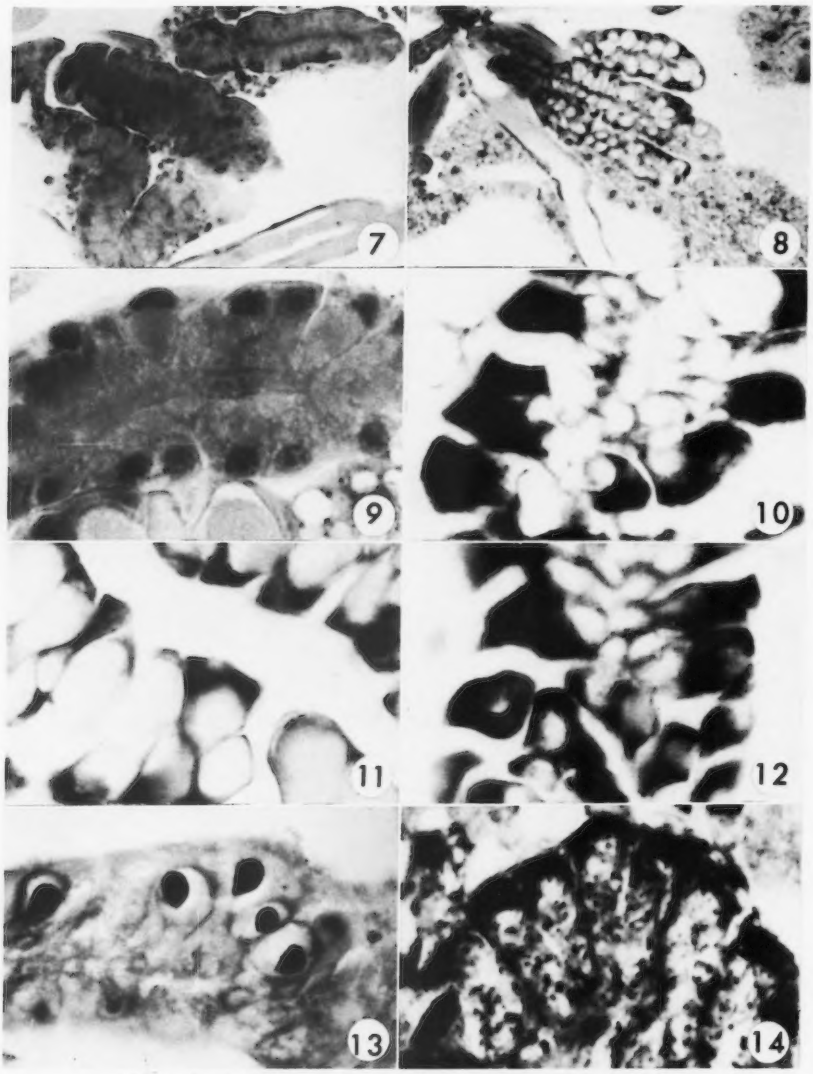
FIG. 2. Female, 72 hours after emergence: Himes's and Moriber's triple stain. (Abbreviations as for Fig. 1.)

FIG. 3. Taenidia in lateral lobe: Himes's and Moriber's triple stain.

FIG. 4. Female, 72 hours after emergence. Neck regions of central and lateral lobes: azan trichrome. (Magnification as Fig. 6.)

FIG. 5. Female, 72 hours after emergence. Central lobe acinar cells with peripheral nuclei: azan trichrome. (Magnification as Fig. 3.)

FIG. 6. Female at emergence showing accessory acinus: azan trichrome.



(Magnification: Figs. 7 and 8, as Fig. 6; Figs. 9–14, as Fig. 3.)

FIG. 7. Female, 36 hours after emergence: azan trichrome.

FIG. 8. Male, 48 hours after emergence: azan trichrome.

FIG. 9. Central lobe acinar cells showing enlarged nucleoli, 24 hours after ingestion of blood: azan trichrome.

FIGS. 10 and 11. RNA staining, 36 and 72 hours, respectively, after emergence: azure B bromide.

FIG. 12. RNA staining, 24 hours after ingestion of blood: azure B bromide.

FIG. 13. PAS-positive globules in neck of lateral lobe.

FIG. 14. Acid protein granules in lateral acinus: methylene blue, pH 8.0.

The Salivary Glands of Male Mosquitoes

We observed no changes in the appearance of the glands of the male mosquito following emergence. The glands are small and trilobed, and seldom extend as far as the mesothorax. The three lobes are approximately of equal length and appear to be similar to each other in morphological and histological detail (Fig. 8). There is no obvious division into a neck and acinus. The anterior portions of the somewhat pear-shaped lobes contain small, darkly staining cells, while the cells of the posterior portions are larger and vacuolated. The whole gland stains lightly and uniformly with PAS. The small size of the male glands has been reported by Shishlyaeva-Matova (24), Metcalf (18), and Jensen and Jones (11).

The Effect of Feeding on the Salivary Glands of the Female

Normal *Aedes aegypti* females will bite and feed by 72 hours after emergence. In accordance with the work of Christophers (4) and Grassi (Nuttall and Shipley (20)) we found no immediate alteration in the cytological picture of the glands at the time of feeding on human hosts. Ingestion of sucrose likewise had no effect. However, when blood-fed females were examined at various intervals after feeding, a pattern of activity was found. By 24 hours the nucleoli had enlarged greatly (compare Fig. 5 and Fig. 9), and an increase in basophilia (RNA) had occurred around the nuclei (compare Figs. 10, 11, and 12). After 24 hours, the nucleoli returned to the "resting state".

Histochemistry of the Mature Gland of the Female Mosquito

The results of the histochemical analyses are given in Table I. The central acinus and the lateral neck are strongly PAS positive (particularly the lateral

TABLE I

Results of histochemical analysis of mature salivary glands of *Aedes aegypti* females*

Stain	Lateral lobes	Lateral necks	Central lobe	Central neck
Carbohydrates				
PAS	++	++++ (globules)	+++	+
Alcian blue (with PAS)	−	−	−	−
Azure A (γ metachromasy)	−	−	−	−
Diastase/PAS		No reduction in staining		
Fats				
Sudan black B	−	−	−	−
Proteins				
Basic proteins	++	+	+++	++
Acid proteins	++	+++	−	
SH groups	++	++	++	++
Amino acids				
Lysine, arginine, histidine	++	++	++	++
Tryptophan	−	−	+++	+
Tyrosine	+	+	+	+
RNA				
Azure B bromide	−	++++	−	−

*Number of plus signs indicates relative intensity of staining.

neck where the staining is concentrated into globules) (Fig. 13), whereas the neck of the central lobe stains faintly, if at all, and the lateral lobes stain moderately. When the PAS stain was used in conjunction with alcian blue at pH 4, there was no evidence of staining with the latter dye. Furthermore, no metachromasy was found in any part of the gland. Finally, no reduction in PAS intensity occurred after diastase treatment to remove glycogen.

In the lateral necks, material that is removed by ribonuclease stains strongly with azure B bromide at pH 4, and is therefore taken to be RNA. No other part of the glands stains with azure B bromide at this stage. The lateral necks also stain heavily with methylene blue at pH 8. It was thought that this might relate to the RNA staining, but treatment with ribonuclease and deoxyribonuclease did not reduce the intensity. Large cytoplasmic granules in the lateral acini also stain with methylene blue at pH 8 (Fig. 14). The general areas of PAS positivity coincided with the areas stained with mercuric bromphenol blue, and the PAS and bromphenol blue areas appeared to be spatially distinct from the granules.

Discussion and Conclusions

The histological findings indicate three principal regions of secretory activity in the mature salivary glands of the female mosquito: the central acini, the lateral acini, and the necks of the lateral lobes. This conclusion was also reached by Shishlyaeva-Matova (24) in her histological studies of *Anopheles sacharovi*.

The histochemical evidence suggests that each of these three areas elaborates a different secretory product, possibly two in the lateral necks and acini. It is probable that the secretory product of the central lobe is a conjugated mucopolysaccharide. A carbohydrate–protein complex may also be one of the products of the lateral lobes, since the PAS and basic protein staining appear to be located in the same region. Furthermore, large acid protein granules are also found in the lateral acini. By analogy with pancreatic granules, it is possible that these protein granules may have some enzymatic function. The continued RNA synthesis occurring in the lateral neck cells of the mature gland, according to the prevailing interpretation, would indicate further macromolecular synthesis. Two types of secretion are found in these lateral neck cells: acid protein, and globules of PAS-positive material. These globules are not removed by diastase, nor do they exhibit any metachromasia. They are probably, therefore, a neutral mucin which is constantly synthesized and may well have a lubricatory function. The acid protein secretion from these cells could also be involved in lubrication. In this regard it is interesting to note that Barth (2) suggested that the lipocrine (lipoid) secretion of the lateral lobe of *A. scapularis* may be the solution vehicle for the rhagiocrine (protein-like) products of the central lobe.

The small size of the nucleoli and lack of RNA in the remaining regions of the glands (lateral acini, central necks, and acini) suggests that they have ceased synthetic activities by 72 hours after emergence. However, 24 hours subsequent to ingestion of blood, the nucleoli of the acinar regions of both lateral and central lobes increase greatly in size. There is a concomitant increase in RNA at this time. It appears likely that feeding depletes the glands

and that this depletion leads to a resynthesis of secretory products.

The salivary glands of the male are strikingly different in size, histological detail, and staining characteristics from those of the female. They do not grow and appear almost degenerate. Since the male mosquito does not feed on blood, the extensive development of the female glands is very likely to be related to the parasitic habit.

The anticoagulin and agglutinin properties ascribed to the saliva of some species of mosquitoes have been reported as absent in *A. aegypti* (Yorke and Macfie (27); McKinley (16)). Apart from these properties, few other functions have been proposed for the saliva of female mosquitoes. The role of the glands is even more difficult to interpret in the light of the findings of Hudson *et al.* (9). They showed that *A. stimulans* (Walk.), when deprived of saliva, were able to digest sucrose and blood normally. Hudson (unpublished) has recently found that an *A. aegypti* female, with the salivary duct cut, was able to feed and to mature and lay a normal number of eggs which hatched and developed into adults. The insect fed again, and again developed a normal number of eggs, seven of which were laid. In general *A. aegypti* will feed quite readily after the salivary duct has been cut. According to this evidence the glands can have no essential role in feeding nor apparently in digestion either. However, Hudson *et al.* (9) have suggested, from subjective criteria, that an anaesthetic component is present in saliva which at least would have some adaptive significance and account partly for the otherwise puzzling sexual dimorphism.

Female mosquitoes are normally ready to feed at approximately 72 hours after emergence and at this time the salivary glands are fully developed. It is tempting to try to relate the initiation of the feeding drive to the maturation of the glands. However, there are numerous reports that younger females may take a blood meal and recently Woke[5] has observed blood feeding by *A. aegypti* less than 24 hours old. In nearly all cases the bites from these mosquitoes produced skin reactions on the human arm or leg. This would indicate that some secretion by the glands occurs before they are fully developed, or, less likely, that in these mosquitoes the glands have reached maturity early. In considering their function we should not overlook the fact that we are still quite ignorant of the full sequence of events which leads to the taking of a blood meal by mosquitoes in nature. Possibly the salivary glands play some essential role in this behavior.

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COMPARISON OF TISSUES FROM SOLID- AND HOLLOW-STEMMED SPRING WHEATS DURING GROWTH

II. FOOD VALUES DETERMINED WITH THE PALE WESTERN CUTWORM, AGROTIS ORTHOGONIA MORR. (LEPIDOPTERA: NOCTUIDAE)[1]

R. KASTING AND A. J. MCGINNIS

Abstract

Lyophilized stem tissues of Rescue and Thatcher wheat were inferior to lyophilized Thatcher sprouts or young leaves as diets for newly hatched or fifth-instar larvae of the pale western cutworm. Pith from solid-stemmed Rescue wheat was an inferior diet to either Thatcher stem or Rescue wall. Most newly hatched larvae lived for periods in excess of 24 days on pith diets but did not molt. Older stem tissues were nutritionally inferior to younger stem tissues. Neither the qualitative nor quantitative composition of 80% ethanol-soluble carbohydrates from the stems, leaves, and sprouts accounted for their relative value as diets for the cutworm. The amino acid composition of ethanol extracts and hydrolyzates of the different plant tissues was generally similar. Some differences were present and therefore amino acid composition cannot, as yet, be ruled out as a factor influencing the diets. Differences in the asparagine content of the plant tissues do not appear to be responsible for their effects on the larvae. There was no simple relationship between the ethanol-soluble nitrogen content of the tissues and their value as diets. The results suggest that a relatively high ethanol-soluble carbohydrate : total nitrogen ratio is an attribute of the tissues that were poor diets for the pale western cutworm.

Introduction

Resistance of wheat to the wheat stem sawfly, *Cephus cinctus* Nort., is associated with the degree of solidness of the stem (11, 13). However, it is not known if this is a result of the pith being nutritionally inadequate for the feeding larvae. Differences in carbohydrate contents of solid and hollow wheat stems (5) and nitrogen contents of stem tissues (4, 9) are not sufficiently large or consistent to explain resistance. It has not been possible to compare directly the nutritive value of stem tissues for larvae of the wheat stem sawfly because the larvae cannot be maintained under artificial conditions (3). Recently it was found that lyophilized and ground plant tissues supported growth and development of another phytophagous insect, the pale western cutworm (8). In an attempt to increase our understanding of plant resistance this method was used for comparing directly the tissues from hollow- and solid-stemmed wheats as food for the pale western cutworm. The qualitative and quantitative composition of some carbohydrate and nitrogen fractions of the tissues were also investigated. In addition, the effects on cutworm development of supplementing Thatcher stem with L-asparagine are included.

Materials and Methods

Plant Material

Two varieties of spring wheat (*Triticum aestivum* L. emend Thell.), Thatcher, a hollow-stemmed variety susceptible to the sawfly, and Rescue, a solid-

[1]Manuscript received February 6, 1961.
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stemmed variety resistant to the sawfly, were each grown in two plots on non-irrigated land at Lethbridge. Plants chosen at random from each plot were pulled from the soil and immediately taken to the laboratory. The first samples were taken July 6–8, when the plants were heading, 55–57 days after seeding. The second samples were collected 7 days later, when the plants were flowering. At that time sawfly larvae are usually feeding in the second and third internodes from the crown. Consequently only these two internodes were processed as described (9) and used in the feeding studies. The pith and wall from Rescue and stems of Thatcher were frozen at −30° C and lyophilized. Usually the tissues were dry within 24 hours. The distillate was retained and the dry plant tissue was ground in a micro-Wiley mill to pass a 40-mesh screen; all samples were stored at −20° C.

Leaf and sprout meals prepared, as described (8), from 10-day-old Thatcher leaves, and 4-day-old Thatcher sprouts were used for control diets and analyses.

Feeding Experiments

Diets of stem tissues were prepared by adding enough of the appropriate distillate to the dry meal to give a final moisture content of 83%. The sprout diet was made up to 67% moisture content by the addition of sprout distillate. Thatcher stem diets supplemented with L-asparagine were prepared by adding asparagine solutions of appropriate concentration to the dry meal in place of stem distillate. Diets containing 83% moisture and 31, 62, 125, and 250 μg L-asparagine per g of moist diet were prepared. Diets were packaged in approximately 1-g portions in aluminum foil and stored at −30° C until fed.

Larvae at two stages of development were used. Newly hatched larvae were obtained from eggs laid by adults collected in the field. Fifth-instar larvae were fed individually through the first four instars on Thatcher wheat sprouts. After the fourth molt the larvae were weighed individually and assigned at random to the various diets. Each diet was fed to 20 first- and 20 fifth-instar larvae except for the L-asparagine-supplemented diets, which were fed only to the newly hatched form. The larvae were handled as described previously (8); growth and development were recorded.

Feeding experiments were conducted with plant material collected during each of 2 years but only the results for the second year, which confirmed and extended the earlier work, are reported.

Chemical Analyses

One gram of the lyophilized plant tissue was extracted with 80% ethanol and the extract was treated subsequently by the method of Murphy (10) for grass samples. The residue left after extraction with alcohol and boiling water was hydrolyzed in 6 *M* HCl for 24 hours, after which the HCl was removed *in vacuo*. The hydrolyzate was taken up in water and the humin was removed by centrifugation. The latter was washed several times. The supernatant solution and washings were combined; water was added to bring the volume to 25 ml.

Soluble Carbohydrates

The total quantities of carbohydrates in the ethanol extracts were deter-

mined in quadruplicate by the anthrone method (10). The qualitative composition of the sugars was determined by paper chromatography (5).

Amino Acids

The nitrogen contents of the dried meals and of the 80% ethanol extracts were determined in duplicate by the micro-Kjeldahl procedure (2).

Since direct chromatography of the alcohol extracts of the plant meals gave unsatisfactory paper chromatograms, the extracts were treated with Dowex-50 in the H^+ form as described by Plaisted (12). The resulting amino acid solutions were evaporated *in vacuo* at 40° C and then the residues were dissolved in 10% isopropanol. Aliquots of these solutions were chromatographed in triplicate on Whatman No. 1 paper previously washed with oxalic acid. The chromatograms were developed first in *n*-butanol : acetic acid : water (4:1:5) and then in water-saturated phenol, pH 12. The supernatant solutions from the hydrolyzed plant materials were similarly chromatographed.

Results and Discussion

The larvae shown in Fig. 1 are representative of those fed for 20 days on diets prepared from lyophilized and ground wheat plant tissues. The larva on the left was fed Thatcher sprouts, which have been shown to be a satisfactory diet for the pale western cutworm (7). The others, from left to right, were fed Rescue wall, Rescue pith, and Thatcher stem collected July 6–8. The stem tissues collected a week later produced generally smaller larvae. The development and survival data in Table I show that Thatcher sprouts were superior to stem tissues as food for newly hatched larvae. Thatcher stems from either collection date and Rescue wall from the early collection allowed significantly better development and survival of larvae than did Rescue wall from the late collection and both piths. However, development of larvae fed on all stem tissues was slow and few insects molted more than twice. The results clearly show that stem tissues of either variety are less satisfactory than sprouts as food for newly hatched larvae and, further, that the pith tissues from Rescue are inferior to stems of Thatcher. Rescue wall tissue from the late collection allowed poorer larval development than did similar tissues from

TABLE I

Development of newly hatched larvae of the pale western cutworm fed on lyophilized wheat tissues

Tissue	% molting			Mean days to molt		% surviving after 24 days
	1×	2×	3×	1	2	
Thatcher sprout	84	79	79	8 ± 1	13 ± 2	84
Thatcher stem: early	43 †	20 †	6	9 ± 2	17 ± 3	33 †
late	26	12	0	10 ± 2	20 ± 3	18
Rescue wall: early	45	31	0	9 ± 2	19 ± 2	36
late	5 †	0	0	14 ± 6	—	5 †
Rescue pith: early	0	0	0	—	—	0
late	5	0	0	—	—	6
Thatcher leaf*	87	73	73	4 ± 1	8 ± 1	73

*Representative data from another experiment (8).
†Not significantly different at 5% level.

TABLE II

Time required to molt and weight gains of fifth-instar pale western cutworm larvae fed on lyophilized wheat tissues

Tissue	Mean weight gain (mg)	Mean stadium length (days)
Thatcher sprout	266	7 ††
Thatcher stem: early	114 †	7
Rescue wall: early	102	7
Rescue pith: early	68 †	8
Thatcher stem: late	49 ††	7
Rescue wall: late	31	10 †
Rescue pith: late	17	10
Thatcher leaf*	173	7

*Data from another experiment.
†Not significantly different at 5% level.

the early collection. A similar trend occurred with Thatcher stems but the differences were not statistically significant (14).

The food value of the plant tissues was also determined for fifth-instar larvae by measuring weight increase and stadium length and subjecting the data to Duncan's multiple range test (14). The superiority of sprouts over the stem tissues as food for fifth-instar larvae is shown in Table II. The gains in weight by the larvae fed the stem diets varied but all were lower ($P < 0.01$) than those of larvae fed the sprout diet. Diets prepared from the early collection of either Thatcher stem or Rescue wall permitted greater weight gains ($P < 0.01$) than did the other stem diets. Tissues collected on the second date allowed consistently poorer development than did the corresponding tissues collected 1 week earlier. Further, on both dates the pith was inferior to either Rescue wall or Thatcher stem as food for the cutworm. Larvae fed wall or pith of Rescue from the late collection gained least weight and required more time to complete fifth-instar development. It appears that weight increase is a more sensitive measure of the value of the diet than is stadium length.

The stem tissues did not support development of either first- or fifth-instar larvae as well as did the sprout diet. It was shown (7) that underfed pale western cutworms developed more slowly and achieved smaller weight gains than did full-fed cutworms. Further studies with the present diets are necessary to determine the part played by consumption. That the diets were consumed by fifth-instar larvae was evidenced by the presence of fecal pellets each day. Hence differences in growth and development of larvae fed the different diets may to some extent be attributed to nutritive value. Rescue pith was inferior to either Rescue wall or Thatcher stem as food for both first- and fifth-instar larvae. This may indicate that pith is deficient in dietary substances necessary for development of the pale western cutworm and may explain some of the resistance of solid-stemmed wheats to the wheat stem sawfly. Furthermore, Thatcher stems did not support normal development of cutworm larvae. In contrast, sawfly larvae thrive in similar field-grown Thatcher stems. Thus it would appear that either the nutritional requirements of the two insects are different or the cutworm is consuming toxic substances from the external portions of the stem that are not normally consumed by the sawfly larvae. If a

PLATE I

FIG. 1. Typical pale western cutworm larvae fed for 20 days on, from left to right, Thatcher sprout, Rescue wall, Rescue pith, and Thatcher stem.

TABLE III

Total and ethanol-soluble nitrogen, and ethanol-soluble carbohydrate contents of wheat tissues

Tissue	Percentage			Ratio	
	Total N	Soluble N	Soluble CHO	Soluble CHO: total N	Soluble CHO: soluble N
Thatcher sprout	4.8	1.9	23.0	4.8	11.9
Thatcher stem: early	1.47	0.31	16.1	11.0	52
late	1.24	0.32	11.5	9.3	36
Rescue wall: early	1.17	0.25	13.1	11.2	52
late	0.81	0.36	12.6	15.6	35
Rescue pith: early	1.04	0.50	25.7	24.7	51
late	0.73	0.65	17.6	24.1	27
Thatcher leaf	5.3	1.9	3.1	0.6	1.6

toxic factor in Thatcher stems is involved, it appears not to be soluble in 80% ethanol since the addition of such an extract to a Thatcher leaf diet* did not affect the development of newly hatched larvae.

Thatcher sprouts supported good larval development and contained large quantities of ethanol-soluble carbohydrates (Table III). Pith tissues were not deficient in these carbohydrates, as they contained more than did the other stem tissues and much more than leaf. The soluble-carbohydrate content of the sprouts was intermediate between that of the piths from the first and second collections. There was a decrease in soluble-carbohydrate content with increasing age of stem tissues but when all the values were considered there was no correlation with the development of the larvae. Fructose and glucose were present in all of the tissues examined and sucrose was present in all but the pith, and appeared in only trace quantities in the sprouts. The poor development of larvae on pith was not due to the absence of either fructose or glucose. A deficiency of sucrose in the pith may have contributed to the poor development but this appears unlikely since sprouts contained only trace quantities of this disaccharide. Moreover, the other stem tissues contained sucrose and yet were obviously poor diets. The foregoing, coupled with the fact that all three sugars were present in Thatcher leaf, and in Thatcher stem and Rescue wall at both dates, indicates that the presence or absence of these compounds is not responsible for the observed growth effects.

Table III shows that the total nitrogen content of the stem tissues was less than one-third that of the leaves or sprouts. The stems also contained less of the soluble nitrogen. Tissues collected at the later date contained less total nitrogen and allowed poorer growth than did similar tissues collected earlier. There is no simple relationship between the soluble-nitrogen contents of the different tissues and their value as diets but the total nitrogen contents appear to be related to dietary value (Tables I, II, III). Nevertheless, the generally poorer development on all the stem tissues is probably not entirely he result of lower nitrogen intake because diets of Thatcher leaf diluted with cellulose powder to even lower nitrogen levels allowed better growth (8).

*Lyophilized Thatcher leaf, 1 part; cellulose powder, 3 parts; water, 8 parts.

The protein : energy ratios influence the nutritional value of diets for chickens and rats (1) and perhaps insects (6). The carbohydrate : total nitrogen ratios, calculated for the various diets (Table III), show that the better diets, sprouts and leaves, had the lowest ratios. The Rescue pith collected on both dates had a higher carbohydrate : total nitrogen ratio than did either the corresponding Rescue wall or Thatcher stems. The other stem tissues had lower carbohydrate : total nitrogen ratios and allowed more growth than did the pith. These results suggest that a high carbohydrate : total nitrogen ratio is associated with a poor-quality diet. However, when the carbohydrate : total nitrogen ratio of a Thatcher leaf diet* was increased from 0.6 to 8 by the addition of an ethanol extract of Thatcher stem there were no deleterious effects on larval growth and development. The development of larvae fed the supplemented leaf diet was much superior to that of larvae fed the Thatcher stem with a ratio of 9.3. While a carbohydrate : total nitrogen ratio of 8 obtained by supplementation was not detrimental to growth and development, the significance of larger ratios such as were found in the pith tissues remains to be determined.

There appears to be no simple relationship between the nutritional value of the diets and the carbohydrate : soluble-nitrogen ratios (Table III). Again the two good diets, sprouts and leaves, have the narrowest ratios but beyond this no definite pattern exists.

Differences in quantities of nitrogen in the stem tissues did not adequately explain their poor nutritional value; however, it was possible that qualitative differences were important. Two-dimensional chromatograms of ethanol extracts of Thatcher sprouts and the different stem tissues from the first date showed that the amino acids present in Thatcher stem were also in the other tissues. These amino acids were alanine, aspartic acid, α-amino butyric acid, asparagine, glutamine, glutamic acid, leucines, phenylalanine, threonine, serine, and valine. In addition the pith contained four other ninhydrin-positive substances; two were tentatively identified as methionine sulphoxide and glycine and the others were unknown. Sprouts differed from pith by the presence of tyrosine and two or more unknown ninhydrin-positive compounds. The extracts from the second collection of stem tissues gave the same relative amino acid patterns.

The asparagine concentration of the sprouts, as estimated from chromatograms, was appreciably higher than that of any of the stem tissues. Likewise Thatcher leaves contained large concentrations of asparagine. Sprouts and leaves are good diets for the pale western cutworm whereas the stem tissues are nutritionally inferior. However, the addition of various concentrations of L-asparagine to the Thatcher stem diet did not improve the value of the food (Table IV).

Two-dimensional chromatograms showed that hydrolyzates of all stem tissues on both dates contained at least the following amino acids: alanine, aspartic acid, glycine, glutamic acid, leucines, methionine sulphoxide, proline, phenylalanine, serine, threonine, tyrosine, valine, and cystine, lysine, arginine, histidine. Hydrolyzates of both sprouts and leaves of Thatcher possessed all

*Lyophilized Thatcher leaf, 1 part; cellulose powder, 3 parts; water, 8 parts.

TABLE IV

Development of newly hatched larvae of the pale western cutworm fed lyophilized Thatcher stem supplemented with L-asparagine

Supplement (μg/g)	No. of larvae	No. molting		Mean days to molt	
		1×	2×	1	2
0	20	3	1	7	12
31	20	0	0	—	—
62	20	5	0	8	—
125	20	2	0	6	—
250	20	5	1	7	12

of the amino acids found in the stem tissues and contained some additional low R_f ninhydrin-positive compounds. These differences in the composition of the tissues may be important in cutworm nutrition.

Conclusions

1. Diets prepared from lyophilized stem tissues of Rescue and Thatcher wheat were inferior to those prepared from lyophilized Thatcher sprouts for either newly hatched or fifth-instar larvae of the pale western cutworm. Poorer growth and development were obtained on pith tissue diets than on either Thatcher stem or Rescue wall diets. Stem tissues harvested 62–64 days after seeding were poorer diets generally than similar tissues harvested a week earlier.

2. The poor development of larvae fed on stem tissues could not be attributed to either the qualitative or quantitative contents of the ethanol-soluble carbohydrates.

3. Low quantities of total nitrogen in the stem tissues did not entirely account for the poor larval growth. Furthermore, there was no simple relationship between the soluble-nitrogen content of the different tissues and their value as diets. Although there were some differences, the amino acid compositions of the ethanol extracts of sprouts, leaves, and stem tissues were generally similar; hydrolyzates of the residues were also similar.

4. A lack of asparagine in the stem tissues was not responsible for the nutritional inadequacy of these diets.

5. A high carbohydrate : total nitrogen ratio is characteristic of the pith of wheat but not of the leaves or sprouts.

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POLYMORPHISM IN THE WHITE-THROATED SPARROW, ZONOTRICHIA ALBICOLLIS (GMELIN)[1]

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Abstract

A sample of 286 museum and 199 live specimens of white-throated sparrows were examined for morphic differences. Two morphic types in both sexes are described according to the color of the median crown stripe. These are the white-striped morph and the tan-striped morph. Regardless of sex, white-striped morphs have more black on the lateral crown areas, less streaking on a wider and grayer chest band, less intense black on the malar markings of the white throat patch and brighter yellow on the superciliary stripe. The proportions of white-striped males and tan-striped females increased from Newfoundland to the Northwest region. White-striped morphs mate selectively with tan-striped morphs. Evidence was presented to show that these morphic types are genetically determined, and once attained, do not change with age. Mechanisms governing polymorphism are discussed in an attempt to explain the existence of the morphic types in the white-throated sparrow.

Introduction

The white-throated sparrow, *Zonotrichia albicollis* (Gmelin), is considered to be a uniform species throughout its range (18). However, during some ecological studies on this species in Algonquin Provincial Park, Ontario, it was noted that considerable variation in the alternate (7) (nuptial) plumage of both sexes existed within the population. It was also noted that the males were not consistently the brighter of the two sexes, as much of the literature leads one to believe. In an attempt to determine the reasons for these variable characters, an investigation was made of a sample of museum specimens from the breeding range of the species. This paper reports on the findings of an examination of museum skins and of live birds handled during a 2-year field study in Algonquin Park.

Materials and Methods

In the museum study, a total of 286 specimens were examined. These study skins were lent by six museums: 134 by the Royal Ontario Museum (ROM), Toronto; 81 by the National Museum of Canada (NMC), Ottawa; 57 by the United States National Museum (USNM), Washington; 6 by the British Columbia Provincial Museum; and 4 from each of the Saskatchewan and Manitoba museums. In order to insure that only birds collected on their breeding grounds and in alternate plumage were used, this sample was restricted to adults collected between May 15 and July 15. In addition, 199 adult birds caught and banded for field studies in Algonquin Park were examined. These birds were caught on territory, and were sexed by the cloacal protuberance (15, 19).

[1]Manuscript received August 31, 1960.
Contribution from the Department of Zoology, University of Toronto, Toronto, Ontario. Field studies were supported by grants to Dr. J. B. Falls from the National Research Council of Canada (1959) and from the Advisory Committee on Scientific Research of the University of Toronto (1960).

In both samples, the color of the median crown stripe, the proportion of black and brown in the lateral crown areas, the markings of the white throat patch, and the intensity of yellow in the anterior part of the superciliary stripe were noted. In the museum sample, the degree of streaking and the intensity of color of the chest band of male birds were examined. For the examination of these last two characters and the color of the median crown stripe, three type series were set up and the birds matched against the types.

In the analysis, the sexes were treated separately. The museum specimens were grouped geographically into the following regions: (1) Newfoundland; (2) Eastern (the Northeastern States, the Maritime Provinces, Labrador, and eastern Quebec); (3) Central (western Quebec, Ontario, and the adjacent United States); (4) Prairie; and (5) Northwestern (northern Alberta, northern British Columbia, Yukon, and District of Mackenzie). Although the data for the live birds were treated separately, they were compared with data for the museum sample from the Central region. Frequency distributions were determined for the color variants of the birds in each group in an attempt to detect any geographic variation or cline in any of the characters tested. For the color variants, the results were treated statistically with the chi square test.

Results

Color of the Median Crown Stripe

The live birds of both sexes examined in Algonquin Park could be readily separated into two groups by the color of the median crown stripe. These two groups were designated arbitrarily as "white" and "tan". In order to determine the degree of variation of the color of the median crown stripe, an examination was made of museum specimens of adult white-throated sparrows in the Royal Ontario Museum collection. A type series of six specimens, representing a gradation in color from light sand brown to white, was chosen. This series consisted of the following specimens: 27908; 76471; 38, 8, 1, 822; 26, 10, 11, 345; 36, 8, 1, 824; and 27902. The first three specimens were "tan" and the last three were "white". There was an obvious gap of at least one grade (grade 4 in the series) between these two groups. Each of the remaining specimens was matched against the series and grouped according to the grade it resembled (Table I). All of the 75 male and 62 female specimens could be classified according to the color of the type series, and none fitted into the gap between the "tans" and the "whites".

The data on the museum specimens confirm the observations made on the

TABLE I

The incidence of seven grades of color of the median crown stripe (from light sand brown in grade 1 to white in grade 7) among adult male and female white-throated sparrows. Grades 1 to 3 and 5 to 7 are represented by type specimens. Grade 4 represents the gap between the two color groups

		Grade						
Sex	*n*	1	2	3	4	5	6	7
Males	75	1	9	14	0	8	11	32
Females	62	3	31	12	0	4	8	4

TABLE II

The number of white-striped (W-s) and tan-striped (T-s) adult male and female white-throated sparrows which exhibit the different percentages of black in the lateral crown areas. The samples are tabulated separately

	Males						Females					
	Museum skins*			Live birds†			Museum skins‡			Live birds§		
% black	W-s	T-s	Total	W-s	T-s	Total	W-s	T-s	Total	W-s	T-s	Total
40	—	—	—	—	3	3	—	10	10	—	10	10
50	—	8	8	—	7	7	—	12	12	—	7	7
60	1	17	18	2	17	19	1	20	21	—	24	24
70	2	43	45	2	9	11	2	14	16	—	6	6
80	16	10	26	8	6	14	6	6	12	3	5	8
90	38	1	39	30	—	30	8	1	9	5	—	5
100	73	—	73	24	—	24	4	—	4	2	—	2
Totals	130	79	209	66	42	108	21	63	84	10	52	62

*$P < .001$.
†$P < .001$.
‡$P < .001$.
§$P < .001$.

live birds in Algonquin Park. The two groups of birds, regardless of sex, therefore are called the "white-striped" morph and the "tan-striped" morph. Associated with these morphic types based on the crown stripe color are certain variations in other plumage characters, which are discussed below.

Percentage of Black on Lateral Crown Areas

The first of the other color variants to be examined was the ratio of black to dark chestnut brown in the lateral crown areas. This character was estimated and expressed as the percentage of the area covered by black (to the nearest 10%). It was found that the variation ranged from 40 to 100% black. The figures were divided into four groups, according to sex and morphic type (Table II). In the white-striped males from the museum sample, the proportion of birds exhibiting the different percentages of black increased to a maximum in the 100% group. This was not true for the other three groups of museum specimens, nor for the groups of live birds from Algonquin Park, in which the percentages of black tend toward a near-normal distribution. The distribution of the percentage groups in each morph was significantly different at the 1% level. Furthermore, in the males of each morph, the modal values for the Algonquin Park birds occur at 10% less black than in the museum birds. However, this difference is not significant. In both live birds and museum skins, and in both sexes, the modes for the two morphs differ by 30% in this character. In the males, a combination of the data for the two morphs gives a bimodal distribution. In both the museum sample and the live birds, the difference in the modes is the same as that between the morphs separately. This is indicative of the existence of two morphs. In the females, a similar bimodal distribution would be expected to occur if the sample of white-striped morphs was larger.

Streaking on Chest Band

The second variant to be examined was the degree of streaking of the chest. This varied from none to as heavy as that found in the song sparrow, *Melospiza melodia* Wilson. A type series representing six grades of streaking was chosen from the museum specimens available. This series consisted of the following specimens: NMC (field number) 59-102; ROM 32837; ROM 18026; USNM 158235; USNM 206294; and USNM 239216, arranged in order of increasing streakedness. Each specimen was matched against the series and grouped according to the grade it resembled. Only the male birds from the museum sample were examined for this character. The numbers of birds in the different grades are shown in Table III. The distribution of the different grades for

TABLE III

The incidence of six degrees of streakedness of the chest band (increasing from none in class 1 to very heavy in class 6) among white-striped and tan-striped adult male white-throated sparrows from the museum sample

		Class					
Morph (males)	*n*	1	2	3	4	5	6
White-striped	85	28	44	7	6	—	—
Tan-striped	52	1	8	18	18	6	1

NOTE: $P < .001$.

the two male morphs are significantly different at the 0.1% level. Therefore tan-striped males have a greater tendency toward streaking than the white-striped males.

Color and Width of Chest Band

Another type series was set up to show the range of intensity of color as well as the width of the chest band. One type series of six grades was used for both characters, since there was found to be a direct correlation between them. This series consisted of the following specimens, which ranged from a wide, dark gray band to a narrow, pale gray band: USNM 158237; NMC (field number) 59-102; NMC 29784; ROM 25, 10, 16, 128; NMC 13361; and USNM 394060. Once again, only the male birds from the museum specimens were used. The frequency distribution for these characters is shown in Table IV. It will be noted that, in the male birds, the tan-striped morphs show a significantly greater tendency toward a reduction in the amount and intensity of pigment in the chest band.

TABLE IV

The incidence of six classes of amount and intensity of pigment of the chest band (from a wide, slate gray band in class 1 to a narrow pale gray-tan band in class 6) among white-striped and tan-striped adult male white-throated sparrows

		Class					
Morph (males)	*n*	1	2	3	4	5	6
White-striped	84	1	16	43	18	6	—
Tan-striped	68	—	4	13	29	19	3

NOTE: $P < .001$.

Pattern of Throat Patch

The variation in the pattern of the white throat patch was examined. Five categories were defined, as illustrated in Fig. 1. The black lines within the throat patch extend from the lower edge of each side of the lower mandible,

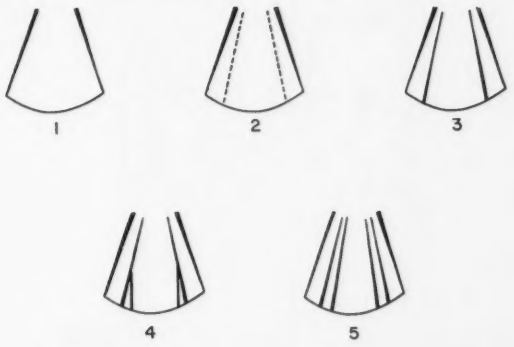

FIG. 1. Diagram of the five types of markings on the throat patch of adult white-throated sparrows. 1, plain; 2, dotted; 3, single bar; 4, forked bar; 5, double bar.

TABLE V

The incidence of five types of throat patterns (from no markings in type 1 to the double-bar pattern in type 5) among white-striped (W-s) and tan-striped (T-s) adult male and female white-throated sparrows. The museum and live birds are tabulated separately

Throat pattern type	Males						Females					
	Museum skins*			Live birds†			Museum skins‡			Live birds§		
	W-s	T-s	Total	W-s	T-s	Total	W-s	T-s	Total	W-s	T-s	Total
1	96	5	101	23	0	23	11	7	18	2	0	2
2	13	14	27	16	0	16	8	7	15	6	4	10
3	5	42	47	1	12	13	1	33	34	2	9	11
4	—	4	4	—	9	9	—	10	10	—	13	13
5	—	—	—	—	1	1	—	—	—	—	1	1
Totals	114	65	179	40	22	62	20	57	77	10	27	37

*$P < .001$.
†$P < .001$.
‡$P < .001$.
§$P < .001$.

TABLE VI

The incidence of different grades of intensity of yellow in the superciliary stripe feathers among white-striped (W-s) and tan-striped (T-s) adult white-throated sparrows. Four categories were used for the museum skins, and five for the live birds

Intensity of yellow	Males						Females					
	Museum skins*			Live birds†			Museum skins‡			Live birds§		
	W-s	T-s	Total	W-s	T-s	Total	W-s	T-s	Total	W-s	T-s	Total
Very bright	—	—	—	48	—	48	—	—	—	7	—	7
Bright	82	1	83	14	2	16	15	1	16	5	—	5
Yellow	22	14	36	3	8	11	6	13	19	—	5	5
Dull	6	24	24	—	15	15	—	15	15	—	10	10
Very dull	—	8	8	—	2	2	—	26	26	—	10	10
Totals	104	47	151	65	27	92	21	55	76	12	25	37

*$P < .001$.
†$P < .001$.
‡$P < .001$.
§$P < .001$.

dividing the patch into three parts. The frequency distribution of these throat types in the museum sample and in the sample of the live birds from Algonquin Park are shown in Table V. This distribution is significantly different at the 0.1% level. It is evident from these data that the tan-striped morphs have heavier throat markings, in both sexes, than the white-striped morphs.

Yellow of Superciliary Stripe

The final plumage variant to be examined was the intensity of yellow coloring of the anterior portion of the superciliary stripe. Four categories were estimated for the examination of the museum specimens. These ranged from bright canary yellow to very dull ocher yellow masked with brown. During the field studies in 1960, the first group was further divided into "very bright" and "bright". The frequency distributions for the four categories in the museum specimens and for the five categories in the live birds are shown in Table VI. The distributions between the morphs of each sample are significantly different at the 0.1% level. The data show that, in both sexes, the white-striped morphs have a brighter yellow on the superciliary stripe than do the tan-striped morphs.

TABLE VII

Proportions of white-striped and tan-striped adult white-throated sparrows in the museum sample from the five geographic regions defined in the text and in the live birds from Algonquin Park. Figures in parentheses represent the number of birds in each group

Region	Males*		Females†	
	White-striped	Tan-striped	White-striped	Tan-striped
Newfoundland	.43 (6)	.57 (8)	.50 (2)	.50 (2)
Eastern	.46 (13)	.54 (15)	.29 (4)	.71 (10)
Central	.63 (68)	.37 (40)	.25 (10)	.75 (30)
Prairies	.71 (22)	.29 (9)	.09 (1)	.91 (10)
Northwestern	.52 (14)	.48 (13)	.44 (4)	.56 (5)
Algonquin Park	.63 (79)	.37 (47)	.22 (16)	.78 (57)

*$P = .10$ (museum sample only).
†$P = .10$ (museum sample only).

Geographic Distribution

Of the several plumage characters examined in the total sample of 286 museum specimens, only the color of the median crown stripe showed geographic variation. Both white-striped and tan-striped morphs could be recognized throughout the breeding range, but the relative proportions of the two morphs indicate a cline from east to west, as shown in Table VII. The males show a clinal increase in the proportion of white-striped morphs from Newfoundland to the Northwest region. This cline is reversed in the females. These clines are not significant at the 5% level. The data for the live birds from Algonquin Park are in agreement with the relative frequencies of the morphs found in the museum sample from the Central region.

Mated Pairs

There is some evidence from the field studies that there is a behavioral difference between the morphs. The morphic types of 110 mated pairs were

TABLE VIII

The morphic types of 110 mated pairs of white-throated sparrows banded in Algonquin Park in 1959 and 1960

Mated pairs			Number of pairs*	
Male	×	Female	Observed	Expected
White-striped	×	White-striped	0	19
White-striped	×	Tan-striped	79	60
Tan-striped	×	White-striped	27	8
Tan-striped	×	Tan-striped	4	23

*$P < .001$.

known, and the distribution of these are shown in Table VIII. It is evident that in all but four cases, mated pairs are composed of opposite morphic types. The probability that mating occurs at random is less than .001. Furthermore, the plumage types of the first and second mates of 15 retrapped birds were recorded. These involved 31 pairs. In all but one case, the plumage types of the second mates were the same as those of the first. These observations are also significant, at the 0.1% level.

Discussion

Descriptions in the literature on white-throated sparrows, including popular bird books, are of two basic types. One group of authors states that the adult males have a black-and-white striped head pattern, a pure white throat bordered by a black or blackish line, and a gray chest. The females are said to be indistinguishable from the males. The second group of authors state that the females are usually duller than the males, and have brown tinging on the crown stripes, black malar markings on the throat patch, and a duller throat patch. It is evident from the observations presented in this paper that not all males have the bright plumage suggested in the literature, and that not all females are duller than males. From the data reported here, two morphic types can be recognized in the white-throated sparrow, and this may explain the confusion that exists in the literature. These are the white-striped and the tan-striped morphs. It was found that the tan-striped morphs of either sex have more streaking on the chest, less black on the lateral crown areas, a narrower and paler chest band, heavier throat markings, and duller yellow in the superciliary stripe.

In order to explain the existence of dull (tan-striped) males, some authors have suggested that the bright plumage is acquired in more than one year (2, 4, 10, 13, 14, 17). There is evidence from the present study and from the literature that the plumage variation described here is not due to an age difference, but once expressed by the first prealternate (7) molt, the plumage is maintained. Some of this evidence comes from the recoveries of banded birds. In the present study, 28 of the birds banded in 1959 were retrapped in 1960. These included 26 birds banded as adults in 1959: 14 male and 3 female white-striped morphs, and 6 male and 3 female tan-striped morphs. The plumage patterns of these birds were the same in 1960 as they were in 1959. The remaining two birds, both females, were banded as juvenals in July and

early August, 1959, respectively. In 1960, one was a white-striped morph, and the other was a tan-striped morph. As the nests of both birds were found in 1960, they had attained sexual maturity in one year. Forbush (4), Brackbill (1), and Nichols (11, 12) reported banded white-throated sparrows which had not acquired a bright plumage after 2, 2 and 3, and $6\frac{1}{2}$ years respectively. It is unlikely that these birds would ever attain a bright plumage. Studies on the white-crowned sparrow, *Z. leucophyrs pugetensis* Grinnell (3), and Harris' sparrow, *Z. querula* (Nuttall) (20), have shown that the alternate plumage is acquired during the first year by the first prealternate molt. Since the white-throated sparrow is closely related to these two sparrows, it does not seem likely that it would take longer than 1 year to achieve its alternate plumage. If this is true, then the two 1-year-old nesting females reported above had their final plumage type. If the plumage differences are not a matter of age as the data reported herein suggest, then it can be concluded that the white-throated sparrow is a polymorphic species.

Polymorphism is defined by Ford (6) as "the occurrence together in the same habitat of two or more distinct forms of a species in such proportions that the rarest of them cannot be maintained by recurrent mutation." As the white-striped morphs mate with the tan-striped morphs (Table VIII), there is no doubt that the two morphs do occur together in the same habitat. An earlier definition given by Ford (5) defines polymorphism as "two or more well marked forms capable of appearing among the offspring of a single female, and occurring with frequencies high enough to exclude the maintenance of the rarest of them by recurrent mutation." This definition is very similar to his later one, but it includes the feature of heterogeneous broods. At present, there is no evidence from studies on white-throated sparrows to support this criterion. However, alternate plumages of complete broods of known parentage are being studied.

Polymorphism has been divided into two basic types, balanced and transient (5, 8, 16). In balanced polymorphism, "two or more forms of the same species are maintained in optimum proportions, a departure from which, in either direction, constitutes a disadvantage" (5). This stability in the ratios of the different forms must be attained by a balance of the selective agencies. No such stability exists in transient polymorphism. As the proportions of the white-striped and tan-striped morphs of the white-throated sparrow show only a slight cline over a wide geographical range, and as there is no difference between the proportions of the two morphs in the Central region collected over a 100-year period and the proportions of the morphs caught in an area of this region during 1959 and 1960, it is safe to assume that this species exhibits a balanced polymorphism.

The color variations in the white-throated sparrow are probably genetically controlled. According to Ford (6), a genetically controlled form occurring in even a small percentage of a population must have been favored by selection. If there is a disruption in the gene pool, then isolation results and new species or subspecies are formed. However, if the different types continue to share the common gene pool, then polymorphism results. As there is selective mating (i.e. white-striped morphs mate with tan-striped morphs), the common gene pool is maintained. Mather (9) states that "once polymorphism is established

in respect to any one character, further characters could be brought into the morphic differences . . . (i.e.) later development built upon earlier structure." The data show that the variation which exists in white-throated sparrows is for several characters which are not absolutely correlated. Therefore, some of these variants might have developed after the first variant was established.

The selective mating of opposite morphs of the white-throated sparrow appears to be unique among bird species. Polymorphism has been recorded in 25 families of birds (8), but no other cases of this type of selective mating have been reported.

Polymorphism may arise when the heterozygote is at a greater advantage than either homozygote (5). The selective mating of opposite morphs of the white-throated sparrow may insure heterozygosity within the population. The mixture of genes through this selective mating may be analogous to heterosis, which often improves the viability of the species (16). This may explain the success and abundance of the species over its large breeding range.

As previously stated, the maintenance of balanced polymorphism depends on the sharing of a common gene pool. According to Mather (9), "cooperation must outmatch competition if the less favoured morphic type is to be held in the population, but the competitive balance will itself determine . . . the relative frequencies of the types." As the mated pairs are composed of the two morphic types, cooperation outmatches competition, and therefore there is a balance between the two morphic types.

It is possible that there is a selective advantage for the tan-striped females throughout most of the breeding range, as this form is less visible against a background of dead leaves, twigs, and soil than the white-striped female is, and would therefore be less subject to predation than the white-striped morph when it is on the nest, which is typically on the ground. However, if the nest is in an area where the nesting cover is lichen (e.g. the reindeer moss, *Cladonia* spp.), which are whitish in color, the white-striped female might have an equal or better survival advantage over the tan-striped female. As the birds tend to mate in opposite morphs, it may be that the nesting cover determines the proportions of male morphs, in a given area. However, this is speculation, and further study will be necessary before any conclusion on the factors governing the morphic types can be made. At present, studies on the genetics and behavior are being conducted by the author. The data from this work will be published at a later date.

Acknowledgments

The author wishes to express his endebtedness to the following persons and institutions for the assistance they gave in this study: Mr. L. L. Snyder of the Royal Ontario Museum for his encouragement, advice, and direction in the collection and analysis of the museum data; Mr. Robert Walker and the author's wife for their help in the recording of the data; Dr. J. B. Falls, Department of Zoology, University of Toronto, for his suggestions in the preparation of this manuscript; Mr. D. B. W. Reid, School of Hygiene, and Dr. D. B. DeLury, Department of Mathematics, University of Toronto, for their advice on the statistical analysis of some of the data; and the National Museum of Canada through the courtesy of Mr. E. W. Godfrey, the United States

National Museum through the courtesy of Dr. H. H. Friedman, and the Provincial museums in British Columbia, Saskatchewan, and Manitoba, through the courtesies of Messrs. C. J. Guiguet, R. W. Nero, and R. W. Sutton respectively, all of which lent white-throated sparrow specimens for this study. Research facilities in Algonquin Park were provided by the Ontario Department of Lands and Forests, through the courtesy of Dr. C. D. Fowle and Mr. R. Stanfield.

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ON SOME TREMATODES FROM THE INTESTINE OF THE MARINE TURTLE, CHELONE MYDAS, FROM THE CARIBBEAN SEA[1]

S. P. GUPTA[2]

Abstract

The following trematodes were recovered from *Chelone mydas* from Trinidad, T.W.I.: *Cricocephalus albus* (Kuhl and Hasselt), *Pleurogonius mehrai* (Ruiz) of the family Pronocephalidae, *Neoctangium travassosi* (Ruiz), *Deuterobaris chelonei* sp. nov. of the family Angiodictyidae, and *Schizamphistomoides chelonei* sp. nov. of the family Paramphistomidae.

The trematodes recorded in this paper were recovered from the intestine of *Chelone mydas* from Trinidad, The West Indies. None had previously been recorded from The West Indies.

Pronocephalidae Looss, 1902

Cricocephalus albus (Kuhl and Hasselt, 1822) Looss, 1899 (Fig. 1)

The numerous specimens available differ from the original description (4) only in being smaller (1.78 to 2.13 mm), and in the possession of a very small oesophagus.

Pleurogonius mehrai Ruiz, 1946 (Fig. 2)

Numerous specimens of this species were present. This trematode has been recorded by Mehra (5) from the same species of host in Karachi under the name of *P. chelonii*. It is very closely related to *P. lobatus* Looss, 1901 (4) and *P. candidulus* (Linton, 1910)(3).

Angiodictyidae Looss, 1902

Deuterobaris chelonei sp. nov. (Figs. 3–5)

Five specimens of this species were present in the collection.

Body elongate, 9.4 to 10.4 mm long, 2.0 to 2.7 mm wide. Head lobe (Fig. 3) 0.25 to 0.4 mm long, 0.2 to 0.28 mm wide, containing oral sucker projecting anteriorly over dorsal surface, oral surface elongate without backwardly directed outgrowths. Oesophagus 0.96 to 1.12 mm long, with pharynx-like swelling posteriorly. Intestinal caeca simple, narrow, not close to lateral margins of body, terminating 1.74 to 1.82 mm from posterior extremity. Genital pore 0.05 to 0.09 mm from anterior extremity, a little behind oral sucker. Excretory pore submedian, with rosette of numerous ciliated diverticulae (Fig. 4), 0.09 to 0.15 mm from posterior extremity. Excretory system consists of a V-shaped vesicle, the reticular part (Fig. 5) consisting of numerous irregular branching channels directed anteriorly. Lymphatic system of five pairs of longitudinal channels with numerous branches anteriorly and to lateral margins of body.

[1]Manuscript received December 16, 1960.
Contribution from the Institute of Parasitology, McGill University, Macdonald College P.O., Que., with financial assistance from the National Research Council of Canada.
[2]Postdoctorate Fellow, National Research Council of Canada. Present address: Department of Zoology, University of Lucknow, Lucknow, India.

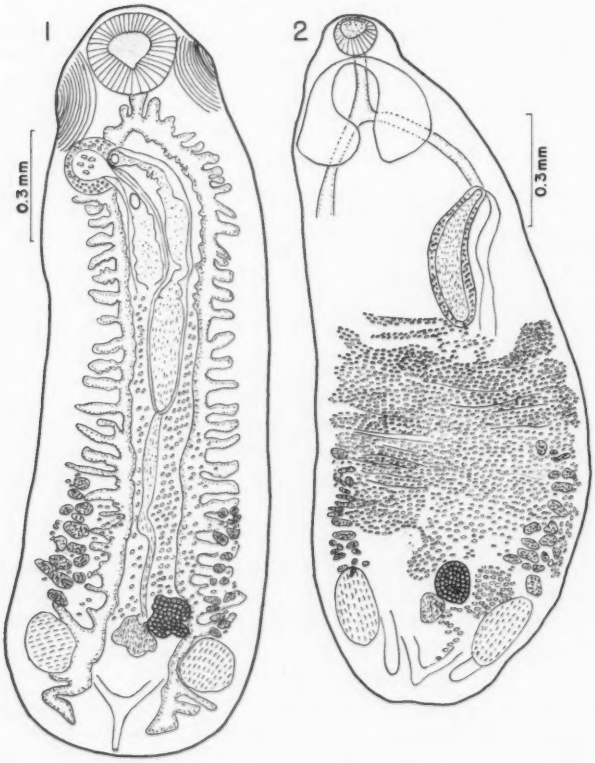
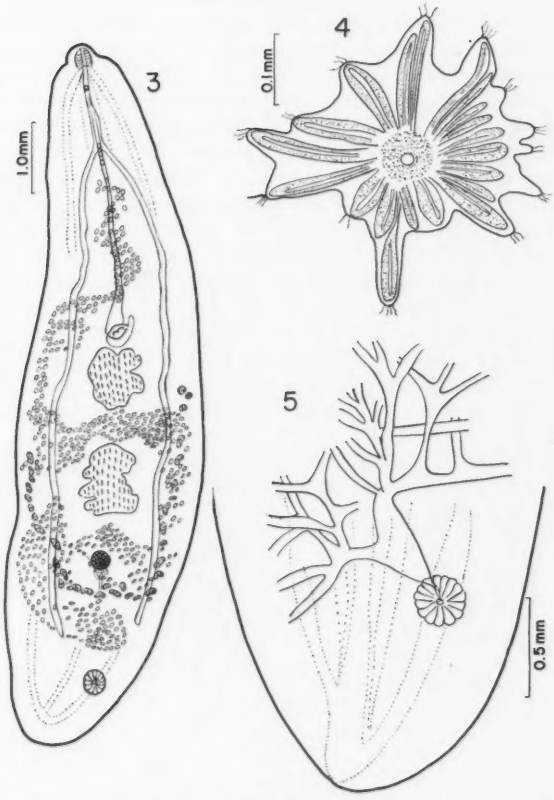


FIG. 1. *Cricocephalus albus* (Kuhl and Hasselt, 1822) Looss, 1899. Dorsal view.
FIG. 2. *Pleurogonius mehrai.* Ventral view.

Testes lobed, tandem, intercaecal, in mid-third of body. Anterior testis 0.78 to 0.9 mm by 0.64 to 0.7 mm in size, 3.72 to 3.57 mm from posterior end. Posterior testis, 0.87 to 1.01 mm by 0.64 to 0.73 mm in size, 0.04 to 0.07 mm apart from anterior testis. Vas efferens arises from outer margin of testis, running forward to open at base of vesicula seminalis which lies close in front of anterior testis. No true cirrus pouch. Pars prostatica elongated, ductus ejaculatorius and metraterm short, all lying free in parenchyma.

Ovary, 0.21 to 0.26 mm by 0.19 to 0.23 mm in size, a little behind hind testis, 2.33 to 2.61 mm from posterior extremity. Vitellarian follicles in U-shaped pattern, extending on left side from level of hind testis to middle of anterior testis on right side, mainly lateral to intestinal caeca, uniting posteriorly to ovary a little anterior to caecal ends. Mehlis gland a diffused structure posterior to ovary. Uterine coils extend posteriorly to hind end of intestinal caeca then wind anteriorly between ovary and posterior testis and between



FIGS. 3–5. *Deuterobaris chelonei* sp. nov. Fig. 3, dorsal view. Fig. 4, "Rippen" dorsal view. Fig. 5, posterior portion, dorsal view.

testes, extending into lateral fields.

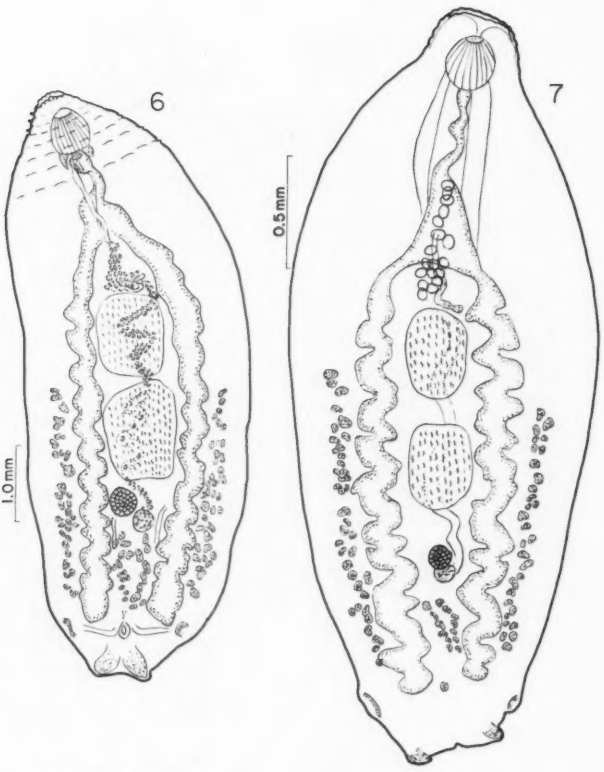
Eggs large, 0.04 to 0.05 mm by 0.03 to 0.04 mm.

Discussion

This species closely resembles the only other species, *D. proteus* (Brandes, 1891) Looss, 1902 (6, 10), recorded from *Chelone viridis*, but differs in having vitelline glands a little anterior to intestinal caeca, five instead of two pairs of lymphatic vessels, and sinuous instead of simple intestinal caeca, and in the absence of two backwardly directed outgrowths on the oral sucker. It is accordingly regarded as new with the specific name of *D. chelonei* sp. nov.

Neoctangium travassosi (Ruiz, 1943) (Figs. 6, 7)

A considerable number of both mature and immature specimens of this trematode were present. They were in general similar to those described originally by Ruiz (8) from *Tartaruga marinha* from Brazil. A few had the



FIGS. 6–7. *Neoctangium travassosi* (Ruiz, 1943). Dorsal views.

anterior testis smaller than the posterior and entire, while the ovary was close to the posterior testis (Fig. 6). Most, however, were smaller (3.51 to 4.52 mm long by 1.3 to 1.82 mm wide) whereas Ruiz's specimens were twice that size. In these specimens also (Fig. 7), the vitelline glands extended anterior to the mid-region of the anterior testis; in Ruiz's specimens they did not extend beyond the anterior edge of the posterior testis. These differences are probably interspecific variations and may be due to the different species of host. However, diverticula to the oral sucker, present in some, were not observed in others. As all the specimens came from a single animal, they are all referred to the same species.

Paramphistomidae Fischoeder, 1901

Schizamphistomoides chelonei sp. nov. (Fig. 8)

Five specimens of this trematode were present.

Length 5.25 to 5.45 mm, width anterior to acetabulum 1.01 to 2.5 mm. Body smooth, elongated; rounded extremities with slight, distinct constric-

tions in mid-region of acetabulum. Oral sucker 0.63 to 0.7 mm long, 0.37 to 0.44 mm wide, elongated with two small diverticula at posterior end. Acetabulum large, 1.27 to 1.39 mm long, 1.04 to 1.2 mm wide, terminal, with ventral aperture at posterior extremity of body. Oesophagus sinuous, about 0.51 to 0.52 mm long, bulb pear-shaped, 0.38 to 0.44 mm by 0.26 mm in size. Intestinal caeca narrow, sinuous, extending to anterior end of acetabulum.

Genital pore median, slightly anterior to intestinal bifurcation.

Excretory pore median, situated dorsally at posterior extremity of body, anterior to acetabulum. Excretory vesicle small, oval, posterior to ovary. Lymphatic system consisting of three longitudinal ducts which extend from anterior region of body to termination of intestinal caeca.

Testes slightly lobed, broader than long, tandem, intercaecal in mid-third of body. Anterior testis 0.49 to 0.52 mm by 0.51 to 0.52 mm in size; posterior 0.44 to 0.49 mm by 0.52 to 0.63 mm in size, 0.09 to 0.17 mm from anterior testis. Cirrus sac 0.21 to 0.26 mm, oval, pretesticular, median. Vesicula seminalis interna completely fills cirrus sac. Unarmed cirrus, 0.21 to 0.22 mm long.

Ovary 0.15 to 0.21 mm by 0.2 to 0.23 mm, postequatorial, oval or spherical, median, about midway between posterior testis and acetabulum. Diffuse

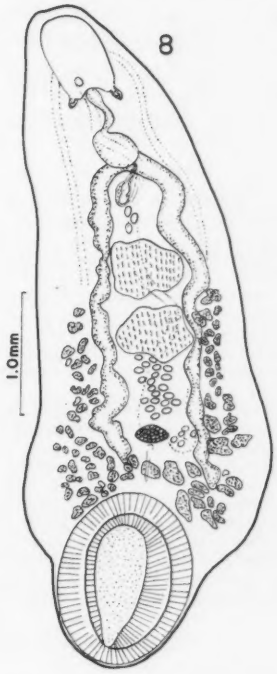


FIG. 8. *Schizamphistomoides chelonei* sp. nov. Ventral view.

Mehlis gland posterior to ovary. Vitelline follicles numerous, small, extending from level of posterior end of anterior testis to anterior end of acetabulum. Vitelline follicles run mostly extracaecal but intruding slightly into post-ovarian area. Uterus transversely coiled between ovary and posterior testis, slightly sinuous in median line, passing dorsal to testes to open into genital pore.

Eggs oval, 0.09 to 0.12 mm by 0.05 to 0.06 mm in size.

Discussion

Four species of this genus have been described from reptiles.

The present form closely resembles *S. spinulosum* (Looss, 1901) Stunkard, 1925 (9), from *C. midas* but differs in the arrangement and extent of vitelline glands, in the position of the genital pore, in its maximum width being anterior to the acetabulum instead of level with it, and in having very sinuous intestinal caeca. It differs from *S. constrictus* (Price, 1936) (7) from *Pelomedusa agaleata* in having testes of irregular shape lying in the mid-third of the body instead of globular shape in the postequatorial region, and in having sinuous intestinal caeca terminating near the anterior end of the acetabulum instead of simple caeca terminating slightly posterior to the ovary. It differs from *S. resupinatus* (Caballero, 1940) (2) from *Dermatemys mawii* in not having pre-equatorial testes in the same level, and from *S. tabascensis* (Caballero and Sokoloff, 1934) (1) from the same host, in not having testes one behind the other in an oblique position. It can also be distinguished from *S. tabascensis* and *S. resupinatus* in the arrangement and extension of the vitelline glands.

Accordingly this species from *Chelone* from Trinidad is regarded as new and given the specific name of *Schizamphistomoides chelonei* sp. nov.

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THE BITING RESPONSE OF LARVAE OF THE PRAIRIE GRAIN WIREWORM, CTENICERA AERIPENNIS DESTRUCTOR (BROWN) (COLEOPTERA:ELATERIDAE), TO VARIOUS EXTRACTS OF GERMINATING RYE SEED[1]

G. R. F. DAVIS

Abstract

Cold-water and hot-water extracts of flour made from germinating rye seed elicited a biting or feeding response from larvae of the prairie grain wireworm. Ethanol and petroleum ether extracts and the residue from the extractions did not. Solutions of L-alanine, L-asparagine, L-aspartic acid, DL-γ-aminobutyric acid, glycine, L-serine, L-threonine, L-tyrosine, or dextrose at a concentration of 0.020 *M* did not act as stimuli for the biting response. Similar concentrations of sucrose and fructose stimulated this reaction weakly. The results suggest that amino acids, polypeptides, or carbohydrates may be the causative agents for the biting or feeding response.

Introduction

Germinating rye seed that was provided alone and in combination with other germinating seeds was fed on to a greater extent than other seeds both by larvae of *Ctenicera aeripennis destructor* (Brown) (2) and by those of *Ctenicera aeripennis aeripennis* (Kby.) (3). Preliminary work in the isolation of the factor that causes larvae to bite rye seed is outlined in this report.

Materials and Methods

Seeds of Antelope rye were germinated for 72 hours at 32° C and 75% relative humidity. The seed was then air-dried at room temperature and ground into flour in a Wiley mill.

A cold-water extract, containing the proteins, amino acids, carbohydrates, vitamin B, and vitamin C of germinating rye seed, was prepared by adding sufficient distilled water to 50 g of this flour to make a total volume of 500 ml. This suspension was allowed to stand at room temperature, with periodic shaking, throughout the day and without shaking overnight. The supernatant was decanted and evaporated to dryness *in vacuo*. This product was redissolved in 25 ml of distilled water and was frozen in the freezing compartment of a household refrigerator.

A hot-water extract, containing polypeptides, amino acids, and the same carbohydrates found in the previous extract, as well as others, was made by adding about 350 ml of distilled water to a second sample of 50 g of flour and by boiling the mixture for 20 minutes. After the mixture had cooled, it was filtered under vacuum and the filtrate was evaporated as before to approximately 30 ml. The resultant liquid was frozen as previously.

The residue from the boiling water extraction was boiled for 15 minutes with 80% ethanol. The mixture was filtered and the filtrate was evaporated as

[1]Manuscript received January 16, 1961.
Contribution No. 78, Canada Department of Agriculture Research Station, Saskatoon, Saskatchewan.

previously to a volume of about 25 ml. The extract was removed to a separatory funnel, the evaporating flask was rinsed with petroleum ether, and this solution was added to that in the separatory funnel. The mixture in the separatory funnel was shaken and allowed to stand overnight. The lower portion of the mixture, containing simple alcohol-soluble proteins, polysaccharides, and vitamin E, was drawn off and frozen as before.

About 250 ml of petroleum ether was added to the residue from the ethanol extraction and the mixture allowed to stand overnight. The liquid was removed by filtration and the petroleum ether phase from the separatory funnel was added to the filtrate. This extract, containing lipids and fat-soluble vitamins, was evaporated to a volume of about 35 ml and was frozen as before.

The residue from the last three extractions was dried in a fume hood, ground in a mortar, and stored in a refrigerator. This product was probably composed only of coagulated protein and insoluble polysaccharides.

To test the effect of various extracts, 10 λ of each extract was placed at a specific location on each of 20 untreated Unidisks (Difco Laboratories Inc., Detroit, Michigan). The residue was tested by wetting another spot on each Unidisk with 10 λ of distilled water and sprinkling on liberal amounts of the residue. Controls were established by moistening one test spot with 10 λ of distilled water and by leaving two other spots untreated. These Unidisks were covered and allowed to dry for 72 hours. The excess residue was shaken off and one Unidisk was placed in each of 20 Petri dishes containing a circle of Whatman No. 3 filter paper, moistened with distilled water. Five larvae of *C. a. destructor* were placed in the center of each dish, the cover was placed on, and the dishes were kept for 72 hours at $20 \pm 2°$ C and $75 \pm 5\%$ relative humidity.

Tests were also conducted with cold-water, hot-water, and ethanol extracts in which each of two extracts were placed on three consecutive test spots on the Unidisk, separated by one untreated, or control, spot on either side. Twenty tests were made for each alternate choice, as before.

Because some workers (1, 4, 6) have indicated that certain amino acids and carbohydrates elicited an orientation or a biting response from other insects, 0.020 *M* solutions of the following were tested against the hot-water extract: *L*-alanine; *L*-asparagine; *L*-aspartic acid; DL-γ-aminobutyric acid; glycine; L-serine; L-threonine; L-tyrosine; sucrose; dextrose; and fructose. A 2% solution of bacteriological peptone was also tested. The same procedure was carried out as in the initial tests, except that the spot containing the hot-water extract was considered the control spot.

After 72 hours, the Unidisks were removed from the Petri dishes, were air-dried at room temperature, and were stored for light-transmission readings with a photovolt densitometer. Because the feeding or biting response of the wireworm larvae caused some of the test spots to turn brown, all spots were bleached in a weak solution of Javex bleach (Javex Company Limited, Toronto, Ontario) before light transmission was measured. The measurement of transmitted light with the photovolt densitometer, despite its limitations, gave a quantitative value in support of the visual assessment of the amount of attack by the wireworms. The values so obtained permitted a statistical treatment and an unbiased comparison of the results.

Results and Discussion

An indication of the response to various extracts by wireworm larvae can be seen in Fig. 1, which is a record of biting activity when all the extracts and the residue were tested. The strong response to the cold-water and the hot-water extracts is clearly evident, as well as the weak response to the ethanol extract.

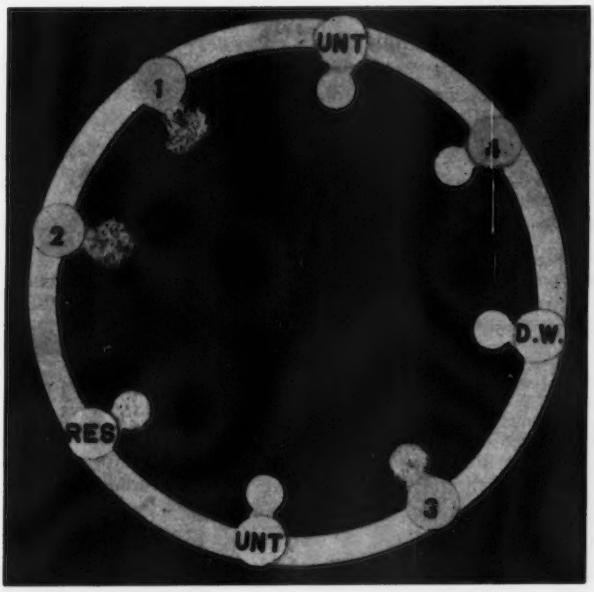

FIG. 1. Unidisk showing amount of attack on each test spot: 1, cold-water extract; 2, hot-water extract; 3, ethanol extract; 4, petroleum ether extract; res, residue; unt, untreated; d.w., distilled water.

The average values for the percentage transmission of light of the various test spots, when the larvae were given a choice of all extracts and the residue, are given in Table I. Both spots on which the cold-water and the hot-water extracts had been absorbed transmitted significantly more light than the others. The low value obtained for the spots treated with the petroleum ether extract is a reflection of the inability to completely bleach this test spot. This is of little importance, because the petroleum ether extract was obviously not too effective in eliciting a biting response from the larvae (Fig. 1).

The average values of percentage transmission of light obtained from tests in which the larvae were given a choice of two extracts are given in Table II. These values show that the stimulant for the biting response is more effective in the hot-water extract than in either the cold-water or the ethanol extract.

When known chemical compounds were tested together with the hot-water extract, none were as effective in producing a biting or feeding response as the hot-water extract. The larvae showed a slight interest in those spots on which

TABLE I

Average percentage transmission of light by test spots when larvae of *Ctenicera aeripennis destructor* were given a choice of all extracts and residue of germinating Antelope rye seed and distilled water

Material tested	No. of spots	Percentage transmission
Control (non-treated)	40	15.5 ± 0.2*
Cold-water extract	20	23.5 ± 4.4†
Hot-water extract	20	23.0 ± 3.1†
Ethanol extract	20	15.9 ± 0.3
Petroleum ether extract	20	13.0 ± 0.2
Residue	20	15.5 ± 0.3
Distilled water	20	15.3 ± 0.2

*Standard error.
†Significantly greater than other values at 5% level of confidence.

TABLE II

Average percentage transmission of light by test spots when larvae of *Ctenicera aeripennis destructor* were given a choice of two extracts of germinating Antelope rye seed

Test	No. spots	% transmission
Cold-water vs. hot-water extract		
Non-treated	40	16.6 ± 0.2*
Cold-water extract	60	35.1 ± 3.0
Hot-water extract	60	44.6 ± 3.2†
Cold-water vs. ethanol extract		
Non-treated	40	16.4 ± 0.1
Cold-water extract	60	39.2 ± 3.1†
Ethanol extract	60	23.9 ± 1.7
Hot-water vs. ethanol extract		
Non-treated	40	15.4 ± 0.1
Hot-water extract	60	20.3 ± 1.1†
Ethanol extract	60	15.1 ± 0.2

*Standard error.
†Significantly greater than other values of group at 5% level of confidence.

solutions of sucrose or fructose had been absorbed, and none in those on which the amino acids, peptone, or other carbohydrates had been placed.

Larvae of three species of *Agriotes* were unaffected by the presence of proteins, but were induced to bite by fatty acids (6). Fatty acids were also effective in orientation responses of adults of two *Limonius* species (5) and in biting responses of silkworm larvae (8). Because the hot-water extract in this investigation was made in such a way as to eliminate most of the protein and because the petroleum ether extract was ineffective in producing biting, both proteins and lipids, as such, may be eliminated as causative agents of biting in larvae of *C. a. destructor*.

Amino acids are responsible for prolonging the feeding of newly hatched larvae of the European corn borer, *Ostrinia nubilalis* (Hbn.) (=*Pyrausta nubilalis* (Hbn.)) on synthetic diets (1). They elicited a biting response from individuals of *Leptinotarsa* sp. (Dr. A. J. Thorsteinson, University of Manitoba,

Winnipeg, Manitoba, personal communication) and an orientation response from elaterid larvae (6). However, the amino acids which have caused orientation or biting in other insects are ineffective for larvae of *C. a. destructor* at a concentration of 0.020 *M*. Other amino acids or different concentrations of the same may, on the other hand, elicit a biting response.

Common plant sugars elicited a biting response from larvae of *Agriotes* spp. (6), from nymphs of the clear-winged grasshopper, *Camnula pellucida* (Scudd.) (Dr. A. J. Thorsteinson, personal communication), and from the silkworm (4). The interest shown by the larvae of *C. a. destructor* to spots treated with solutions of sucrose or fructose suggests that these substances, in different concentrations, may stimulate feeding in wireworms as they do in the silkworm. However, the possibility of glucosides as causative agents of the biting response must also be considered, because they are responsible for the establishment of *Pieris brassicae* (L.) and *Pieris rapae* (L.) (7), and *Protoparce sexta* (Johan.) and and *Leptinotarsa decemlineata* (Say) (9), on their respective host plants.

The present studies indicate that the substance in germinating rye seed which elicits a biting response from larvae of *C. a. destructor* is water soluble. The results suggest also that this substance may be more soluble in hot than in cold water. It is probable that amino acids, polypeptides, or carbohydrates may be responsible for the response obtained in these investigations.

Acknowledgments

The Antelope rye seed used in this study was supplied by the Field Husbandry Department, University of Saskatchewan, Saskatoon, Saskatchewan. The untreated Unidisks were the generous donation of Difco Laboratories, Incorporated, Detroit, Michigan. The technical assistance of Mr. F. T. Eves is also acknowledged.

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THE SIGNIFICANCE OF PECTINASE IN PLANT PENETRATION BY APHIDS[1]

J. W. McAllan and J. B. Adams[2]

Abstract

The feeding punctures of 36 examples of forms of 19 species of aphids on their host plants were examined histologically. The types of plant penetration by the aphids' stylets were compared with the occurrence of pectinase in the saliva of these insects. In 17 cases the aphids penetrated only between cells, and all these insects possessed pectinase; in 9 cases the aphids penetrated directly through cells, and all these aphids lacked pectinase. In five instances aphids possessing pectinase penetrated directly through cells; and there were five examples, all possessing pectinase, that penetrated both between and through cells in the same probe. No aphid lacking pectinase penetrated between cells in the pectic middle lamella. It is suggested that pectinase aids intercellular penetration by its hydrolytic action, but that the enzyme is not necessary when the aphid penetrates directly through cells.

Introduction

In studies of the feeding punctures of Hemiptera, several workers (3, 4, 5, 6, 8, 9) found that certain species penetrated plant tissues between the cells and that others passed directly through cells; some of the observations (3, 5, 6) suggested the presence of a hydrolytic enzyme in the saliva of aphids. Since then we demonstrated the presence of pectinase in the saliva of some aphids (1, 2, 7). In our studies we examined apterous (wingless) and alate (winged) forms, on the hypothesis that these forms might differ physiologically as well as morphologically. We found that they often differed in enzymic content (2), which raised the question of the function of pectinase in plant penetration by aphids. In the present paper we show the association that exists between the type of penetration of plant tissues by aphids and the occurrence of pectinase in their saliva.

Methods

Samples of aphids feeding on various hosts and in different seasons were collected and fixed immediately in the field. Pieces of leaves or twigs, of about 0.5 cm^2 surface and having a moderately "dense" aphid population (15 to 30 aphids) were cut and quickly immersed in a fixative solution consisting of 5 ml of 40% formaldehyde, 2.5 ml of glacial acetic acid, and 20 g of chloral hydrate in 100 ml of water (12). Most aphids remained in a feeding position after fixation, but many were washed off the plant material during subsequent treatment. Materials were fixed for 18 hours, dehydrated, cleared, and embedded. Sections of 10 μ thickness were stained lightly in fast green and safranin O, and examined using phase contrast.

In these sections it was possible to determine whether the stylets passed between or through the cells by observing the shape of the salivary sheath left

[1]Manuscript received February 3, 1961.
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TABLE I

The type of plant penetration by several species and forms of aphids and the occurrence of pectinase in the saliva of these aphids

Species of aphid	Host	Date collected	Form of aphid	Occurrence of pectinase	Type of cell penetration
Amphorophora rubi Kltb.	*Rubus parviflorus* Nutt.	24-VI-59	Apterous	+	Between
Aphis fabae Scopoli	*Tropaeolum* sp.	12-VIII-59	Apterous	+	Between
" " "	*Tropaeolum* sp.	12-VIII-59	Immature alate	+	Between
" " "	*Tropaeolum* sp.	12-VIII-59	Alate	+	Through
" " "	*Philadelphus* sp.	8-VII-59	Apterous	+	Between
" " "	*Philadelphus* sp.	8-VII-59	Immature alate	+	Between
" " "	*Philadelphus* sp.	8-VII-59	Alate	+	Between
Aphis nasturtii Kltb. (=*abbreviata* Patch)	*Solanum tuberosum* L. (g)*	VII-57	Apterous	+	Between
" " "	*Solanum tuberosum* L. (g)	5-IX-57	Alate	−	Through
Aphis oestlundi Gill.	*Oenothera* sp.	5-IX-57	Apterous	+	Between
Aphis pomi DeGeer	*Malus* sp.	2-VI-59	Apterous	+	Between
" " "	*Malus* sp.	2-VI-59	Alate	+	Between
Aulacorthum solani (Kltb.)	*Solanum tuberosum* L. (g)	VIII-57	Apterous	+	Between
" " "	*Solanum tuberosum* L.	IX-58	Alate	−	Through
Cinara curvipes Patch	*Abies balsamea* L.	13-IX-56	Apterous	+	Through
Dactynotus sp.	*Chrysopsis* sp.	10-VII-58	Apterous	+	Through
Eriosoma americanum (Riley)	*Ulmus americana* L.	2-VI-59	Apterous, stem mother	−	Through
" " "	*Ulmus americana* L.	2-VI-59	Apterous, immature	+	Between
" " "	*Ulmus americana* L.	2-VI-59	Apterous adult	+	Both types

TABLE I—(*Concluded*)

Species of aphid	Host	Date collected	Form of aphid	Occurrence of pectinase	Type of cell penetration
Macrosiphoniella millifolii (DeGeer)	*Achillea* sp.	16-VIII-57	Apterous	−	Through
Macrosiphum (*Sitobium*) *avenae* Fab.	*Avena sativa* L.	24-VI-59	Alate	+	Both types
Macrosiphum euphorbiae (Thom.) (= *solanifolii* Ashm.)	*Solanum tuberosum* L. (g)	10-VII-57	Apterous	+	Between
" " "	*Solanum tuberosum* L. (g)	12-VI-59	Alate	+	Through
Myzus cerasi (Fab.)	*Prunus pennsylvanica* L.	8-VIII-58	Apterous	−	Through
" " "	*Prunus pennsylvanica* L.	2-VI-59	Alate	−	Through
Myzus persicae (Sulz.)	*Solanum tuberosum* L. (g)	8-VIII-58	Apterous	+	Both types
" " "	*Solanum tuberosum* L. (g)	8-VIII-58	Alate	+	Both types
" " "	*Brassica napobrassica* Mill. (g)	8-VIII-58	Apterous	+	Both types
Neosymdobius annulatus Koch	*Betula pendula* Roth.	19-VI-58	Apterous	+	Between
Pemphigus betae Doane	*Beta vulgaris* L. (g)	II-57	Apterous	+	Between
Periphyllus negundinis (Thom.)	*Acer negundo* L.	16-VIII-57	Immature alate	+	Through
Phorodon humuli (Schrank)	*Humulus lupulus* L.	12-VIII-57	Apterous	−	Through
" " "	*Humulus lupulus* L.	12-VIII-57	Alate	+	Between
" " "	*Prunus pennsylvanica* L.	12-VI-59	Apterous	+	Between
" " "	*Prunus pennsylvanica* L.	24-VI-59	Alate	−	Through
Rhopalosiphum maidis (Fitch)	*Zea mays* L.	11-VIII-59	Apterous	−	Through

* (g) = aphids from continuing greenhouse parthenogenetic cultures.

in the tissue. Within cells the salivary sheath has a larger diameter and an irregular surface, while if the stylets penetrate either through cell walls or in the middle lamella the sheath is narrower and regular in outline. Van Hoof (11) also distinguished the mode of penetration by this method in studies of the punctures of *Myzus persicae* (Sulz.) and *Aphis fabae* (Scop.) on hyacinth.

Determinations of pectinase in aphid saliva were made by a chromatographic method described previously by the authors (1). Some of the records of the determination of pectinase in aphid saliva presented here have appeared before (2) but are included to associate enzymic content with the type of tissue penetration by the aphid concerned.

Results and Discussion

Thirty-six samples embracing 19 aphid species were examined. Table I summarizes the results of this work and associates the types of penetration of plant tissue with the occurrence of pectinase in different forms of species of aphids. Because the particular form of an aphid on a particular host at a particular date may be, and in several cases appears to be, a physiological entity, we have included in the table all information pertinent to these considerations.

The aphids represent genera with different feeding sites; most feed on the leaves and stems of their hosts. Other examples include *Cinara curvipes* Patch feeding on the needles and stems of *Abies balsamea* (L.) Mill., *Macrosiphoniella millifolii* (DeGeer) on the petioles of *Achillea* sp., *Pemphigus betae* Doane from the roots of *Beta vulgaris* L., *Periphyllus negundinis* (Thom.) from the leaves and seed pods of *Acer negundo* L., and *Eriosoma americanum* (Riley) from gall tissue on *Ulmus americana* L.

But irrespective of the site of feeding or the genus of aphid, the stylet tracks in the host tissue fall into a few distinct types, and all the examples within a type are remarkably similar. In 17 cases the aphids penetrated only between the cells and all of these insects possessed pectinase (Fig. 1A). In nine cases the aphids penetrated directly through cells and all these aphids lacked pectinase (Fig. 1B). In five instances aphids possessing pectinase penetrated directly through cells (Fig. 1C); and there were five examples of aphids, all possessing pectinase, whose stylets passed both between and through cells in the same probe (Fig. 1D). In no case did an aphid lacking pectinase penetrate in the middle lamella. From these results we have concluded that there are two means of tissue penetration by aphids, one mechanical and the other hydrolytic. In agreement with Snodgrass (10) we believe that mechanical penetration is

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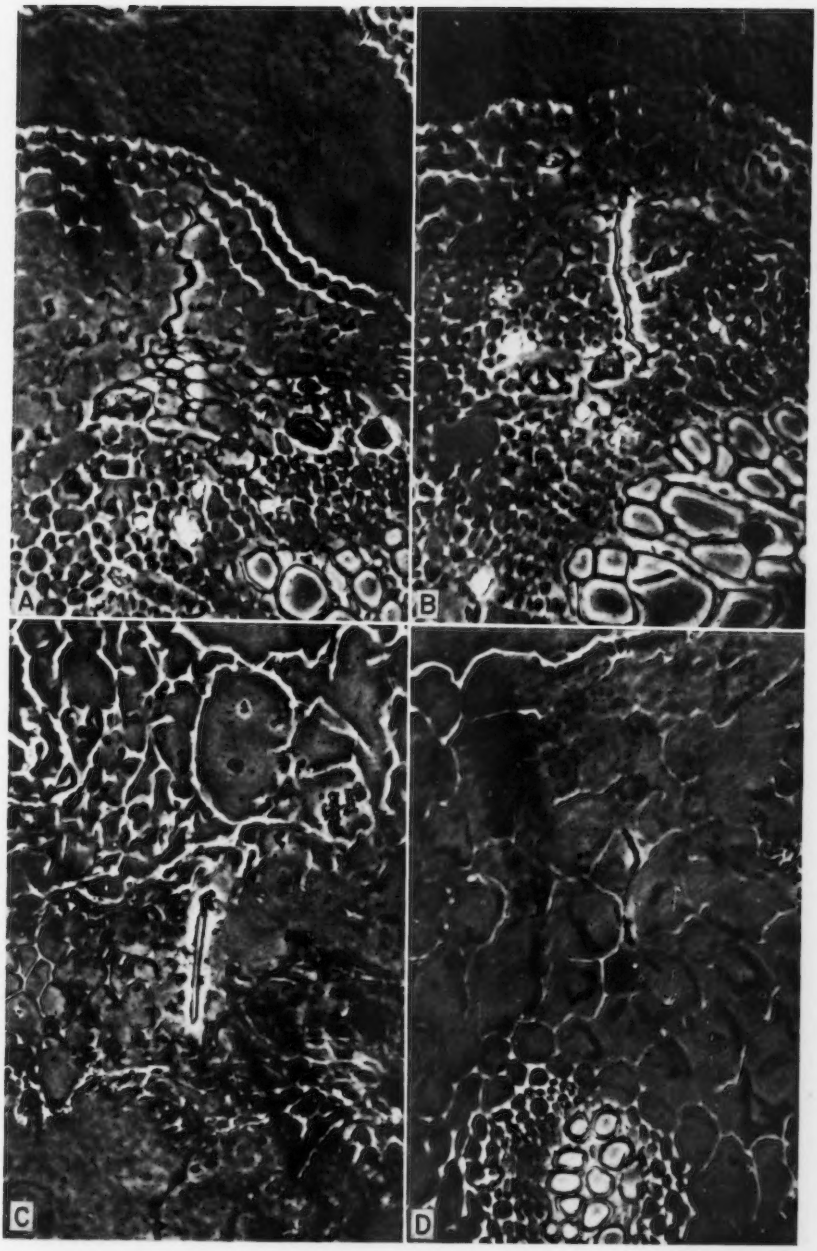
FIG. 1. Photomicrographs of plant tissues showing the four types of plant penetration by aphids' stylets. Magnification, 400×, phase contrast.

A. Penetration between cells by an aphid possessing pectinase. *Aphis pomi* DeGeer, apterous, on *Malus* sp.

B. Penetration through cells by an aphid lacking pectinase. *Phorodon humuli* (Schrank), apterous, on *Humulus lupulus* L.

C. Penetration through cells by an aphid possessing pectinase. *Dactynotus* sp., apterous, on *Chrysopsis* sp.

D. Penetration both between and through cells by an aphid possessing pectinase. *Myzus persicae* (Sulz.), apterous, on *Solanum tuberosum* L.



McAllan and Adams—Can. J. Zool.

the basic means upon which may be superimposed the hydrolytic action of salivary pectinase, acting to soften and render soluble the middle lamella. It appears that these two means may occur separately or jointly. Aphids possessing pectinase may use both means of penetration like the omniphagous species, *M. persicae*. The few samples that lacked pectinase have always been found puncturing directly through the cells.

We have examined our data to learn if the host plant has any effect on the type of tissue penetration by aphids. In this study we were able to compare the four much studied potato-infesting species, *Aphis nasturtii* Kltb., *Myzus persicae* (Sulz.), *Aulacorthum solani* (Kltb.), and *Macrosiphum euphorbiae* (Thom.) on *Solanum tuberosum* L. All these aphids possessed pectinase in the apterous form and penetrated between the cells; however, both forms of *M. persicae* possessed pectinase and went both through and between the cells; and the alate form of *A. solani* lacked pectinase and penetrated only through the cells. The host plant was the same for all.

At different seasons *A. fabae* (Scop.) has been examined from different hosts, its "primary" *Philadelphus* sp., and its "secondary", *Tropaeolum* sp. Regardless of this change in host all these forms of *A. fabae* contain pectinase and penetrate between the cells of either host, with the exception that the alate form on *Tropaeolum* sp., with pectinase, goes through the cells. In contrast to this we find *Phorodon humuli* (Schrank) on its "primary" *Prunus pennsylvanica* L. containing pectinase in the apterous form and puncturing between the cells; lacking pectinase in the alate form and puncturing through cells. The same aphid on its "secondary" host, *Humulus lupulus* L. performs in the reverse, the apterous form lacks pectinase and penetrates through the cells; the alate form contains pectinase and penetrates between the cells.

In our records the host species does not seem to be associated with the type of penetration; the only constant relationship is between the occurrence of pectinase and the type of plant penetration by aphids.

Acknowledgments

The authors express their thanks to Dr. M. E. MacGillivray for many helpful suggestions regarding the host plant relationships of the aphid species and for making the aphid identifications.

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THE EFFECT OF PREY DENSITY ON PREY CONSUMPTION AND OVIPOSITION IN ADULTS OF TYPHLODROMUS (T.) OCCIDENTALIS NESBITT (ACARINA: PHYTOSEIIDAE) IN THE LABORATORY[1]

D. A. CHANT

Abstract

Laboratory experiments indicated that the oviposition and prey consumption rates of *Typhlodromus* (*T.*) *occidentalis* Nesbitt depend on the number of prey available to the predator. The prey species used was *Tetranychus telarius* (L.).

It has long been recognized that with some adult predaceous organisms, while prey consumption is a function of the prey that are immediately available, oviposition is often related to the nutritional history of the organism, particularly when immature, and is relatively unaffected by daily variations in food availability at the time of oviposition. It was suspected that with predaceous phytoseiid mites, while consumption might well be a function of prey density, fecundity was relatively unaffected by any but the very recent nutritional history. Rearing observations led to the hypothesis that the oviposition rate of female phytoseiids is greatly influenced by the amount of suitable food she receives when ovipositing and probably not greatly by the food received when immature. Experiments to test the first part of this hypothesis are reported in this paper.

The Phytoseiidae are of interest because of their possible value in controlling harmful phytophagous mites. A review of ecological and biological studies was given earlier (2). The species chosen for the present study was *Typhlodromus* (*T.*) *occidentalis* Nesbitt. It occurs in small numbers in Southern Ontario on a few herbaceous plants and is common in British Columbia on orchard trees and other deciduous and herbaceous plants (1). The initial colony of mites used in the present work were obtained from British Columbia in 1957.

Methods

Unpublished observations showed that *T.* (*T.*) *occidentalis* females lay more eggs when fresh plant material is available than when it is not, at comparable prey densities. Because of this, and because *T.* (*T.*) *occidentalis* and its prey are found on bean leaves in nature, the present experiments were done on bean leaves. To reduce the area of foliage involved, squares of leaf 2 in. by 2 in. were used and these were kept by the flotation culture method on disks of soaked paper towelling on water-saturated absorbent cotton in petri dishes. Distilled water was used throughout.

The prey species *Tetranychus telarius* (L.) was chosen for this study. It is a

[1]Manuscript received January 31, 1961.
Contribution from Research Laboratory, Research Branch, Canada Department of Agriculture, Vineland Station, Ontario. This work was done while the author was on the staff of the Entomology Research Institute for Biological Control, Belleville, Ontario. Present address: Research Laboratory, Vineland Station, Ontario.

normal prey of *T.* (*T.*) *occidentalis*. For uniformity, only active protonymphs of the prey were offered and these were obtained from healthy stock that was reared on bean plants under the same controlled conditions that the experiments were performed. The predators used were healthy, apparently normal, newly matured females selected at random from stock cultures also reared under the same controlled conditions. Each predator had the same nutritional history in that prey was freely available on fresh bean leaves from hatching to maturity, though it is not known if each used the available facilities to the same extent.

The rate of predation and oviposition of the predators were determined at the following prey densities on the 4 sq. in. of bean leaf surface: 0, 1, 2, 5, 7, 10, 12, 15, 20, 25, and 30. At each density, five squares of leaf, each with a predator, were used and the graphs are based on the averages for these five specimens. The leaf squares were examined daily at the same hour, the numbers of predator eggs and of prey counted, and the female predators transferred to fresh leaves and given fresh prey. The predators were restless at low prey densities and sometimes fell in the water that surrounded the leaf squares. When this happened new predators from the stock cultures were substituted. The experiments were continued for 14 days at each density and, as it was not possible to run these concurrently because of the time involved each day, a possible, though probably unimportant, source of variation was introduced.

The stock cultures and the experiments were maintained at a constant temperature of 74° F.

Results and Discussion

Figure 1 shows that the number of eggs laid by the predator increased with increasing prey density to a maximum average of about 1.9 eggs per day at a density of 20 prey per 4 sq. in.

Figure 2 shows that the consumption of prey increased with increasing prey density to a maximum average of about 11.3 per day at a prey density of 25 per 4 sq. in. The increase of prey consumption from 10.3 to 11.3 and 11.1 at prey densities of 20, 25, and 30 had little apparent effect on the number of eggs laid per day and it seems that 1.9 eggs are about the most that *T. occidentalis* produces under the conditions of this experiment.

Figure 3 shows that, though the predator had a capacity of about 11 protonymphs of *T. telarius* per day, it was not completely efficient at prey levels greater than one and it required a density of 25 prey to achieve its full capacity. The experiment was weighted in favor of the predator because the leaf squares were much smaller than normal leaves (and hence the predator's problem of finding prey was much simplified) and because the prey provided were all of a favored size and stage.

These data, then, clearly indicate that in this experiment the number of prey eaten by the predator was a function of the number of prey provided; and the number of eggs laid per day was a function of the number of prey eaten (and hence of the number available) up to the maximum possible. The maximum rate of oviposition may have been reached slightly before the maximum consumption. The data further indicate that attempts to determine the oviposition rate of this and similar predators are of dubious value since the rate is variable

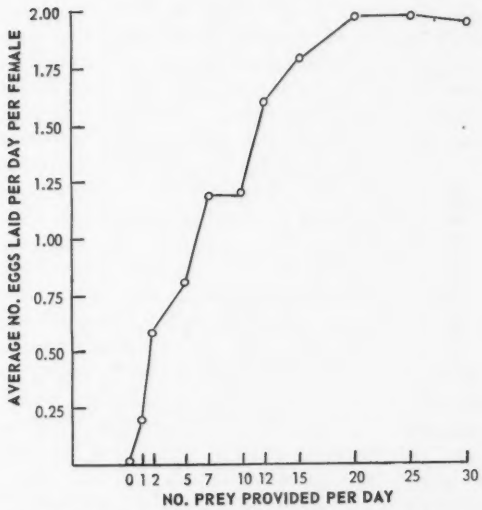

FIG. 1. The numbers of eggs laid by an adult female of *Typhlodromus* (*T.*) *occidentalis* relative to the number of protonymphs of *Tetranychus telarius* provided as food.

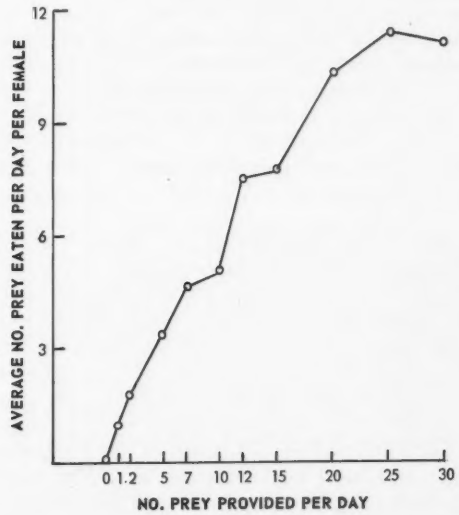

FIG. 2. The numbers of prey consumed by an adult female of *Typhlodromus* (*T.*) *occidentalis* relative to the number of prey (protonymphs of *Tetranychus telarius*) provided.

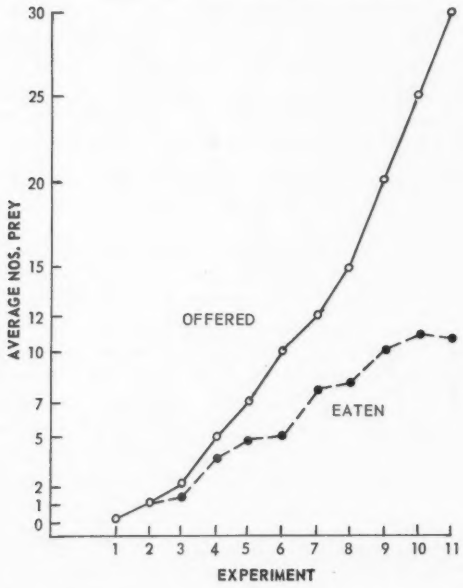

FIG. 3. The efficiency of *Typhlodromus* (*T.*) *occidentalis* as a predator of *Tetranychus telarius* protonymphs on 2 in. × 2 in. pieces of bean leaves.

and depends on the availability of prey, and undoubtedly on other factors as well. The critical statistic in ecological studies on predators of this sort is the minimum prey consumption required for oviposition and the maximum oviposition and prey consumption possible under the prevailing conditions. The present experiments demonstrate that *T.* (*T.*) *occidentalis* requires very little animal food for oviposition; in the present instance the consumption of even one small prey per day permitted the laying of a few eggs, about one every 5 days.

One should be cautious in applying data of this sort to field conditions. It would be folly, for example, to survey prey densities in the field and from them to calculate the number of eggs that *T.* (*T.*) *occidentalis* could lay on the basis of the data herein. These experiments were done only to test the hypothesis that the number of eggs laid per day by a female of *T.* (*T.*) *occidentalis* is influenced by the number of prey consumed by her during oviposition. This proved to be correct and a principle with regard to this species was thereby established.

The possibility that the nutritional history of the predator when immature affects egg-laying by the adults is not ruled out. My experience indicates, however, that approximately the same number of prey are needed for development of the immature mite, regardless of the rate of consumption, and this in turn suggests that food is used mostly to mature and molt and is not stored for later use by the adult.

There was remarkably little variation in the daily number of eggs laid by any one individual at a given prey density, though there was considerable variation in the oviposition rates of different individuals. A period of acclimatization was not found to be necessary when the predators were removed from the stock culture and placed with a certain density of prey; the predators reacted to the new level of food, whether it was higher or lower than that in the stock, within the first 24 hours of the experiment.

Kuenen (3) interpreted several widely accepted generalities in terms of mites and their predators. He maintained that a predator has a maximum ability to consume its prey that cannot be exceeded regardless of how dense the prey may become. He termed this q, and the prey density at which q is possible, b. Each predator has a minimum requirement for prey on which it can remain alive and this was termed p. The minimum density of prey at which the predator can find food was termed a. The present data on daily consumption of prey in relation to prey density provide factual support for Kuenen's conclusion and the values of q, p, a, and b in the present instance of *T.* (*T.*) *occidentalis* preying on *T. telarius* at 74° F on excised leaves are: 11, less than 1, less than 1 per 2 sq. in., and 25 per 2 sq. in., respectively, on a daily basis.

This, together with the data on plant feeding by phytoseiids given in an earlier paper (2), provides an explanation of why these predaceous mites are frequently found developing and reproducing on leaves apparently devoid of prey or with very low densities of prey.

This experiment shows conclusively that reproduction in *T.* (*T.*) *occidentalis* can vary in response to fluctuations in the density of the prey *T. telarius*. This density-dependent reaction, however, could operate only until the predator reached its capacity for oviposition and would then cease to operate. The implications of this, if supported by field observations, are obvious; at prey densities greater than those permitting maximum predator oviposition, the prey would be freed from the density-dependent check imposed by the predator unless the immature predators that hatched from the eggs could mature and start to reproduce at a rate faster than that of the prey. This will be discussed in later papers.

Acknowledgments

The technical assistance of Mrs. P. A. Hutchinson at the Belleville Institute is sincerely and gratefully acknowledged. Also, I am grateful to Mr. C. V. G. Morgan, Research Station, Research Branch, Canada Department of Agriculture, Summerland, B.C., for providing the initial predator colony.

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STUDY OF TWO FILARIOID NEMATODES, CHANDLERELLA CHITWOODAE N. SP. FROM PADDA ORYZIVORA (L.) AND PROTOFILARIA FURCATA CHANDLER, 1929[1]

ROY C. ANDERSON

Abstract

Chandlerella chitwoodae n. sp. from the lungs of *Padda oryzivora* (L.) is described and compared with closely related species. It is similar to *Skrjabinocta petrowi* Tschertkowa, 1946 transferred herein to *Chandlerella* Yorke and Maplestone, 1926. A brief description of paratypes of *Protofilaria furcata* Chandler, 1929 is given and several features of this species are reported for the first time.

Introduction

Eufilaria mcintoshi was previously reported from a Java sparrow imported into Ontario (2). Recently a second filarioid belonging to *Chandlerella* Yorke and Maplestone, 1926 was found in another Java sparrow. This species also seems new but is close to *Skrjabinocta petrowi* Tschertkowa, 1946 (7), the classification of which has been problematic. The new species is named in honor of Mrs. M. B. Chitwood of the United States Department of Agriculture.

Protofilaria furcata, type of its genus, was incompletely described (4), with the result that it was difficult to place with confidence in the classification of the Filarioidea. Through the courtesy of Dr. Allen McIntosh and Mr. W. W. Becklund of the United States Department of Agriculture it was possible to study a male and female specimen from the paratype collection.

Description of Species

1. *Chandlerella chitwoodae* N.SP.

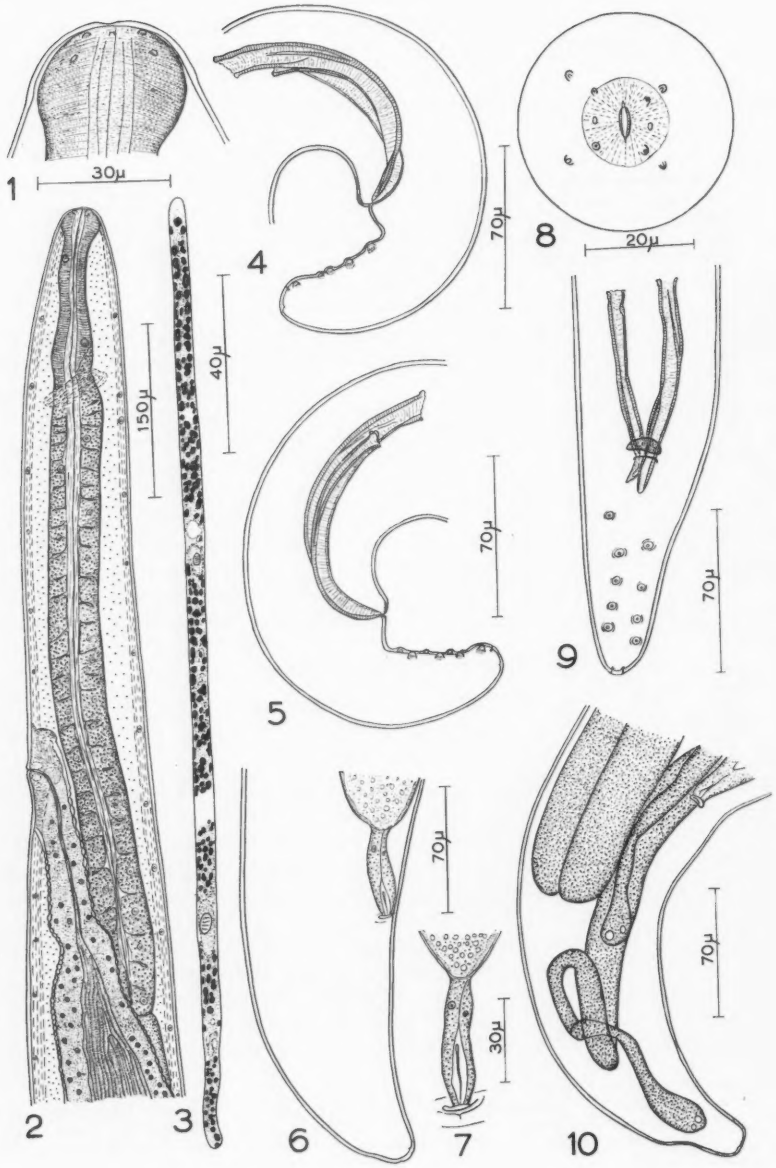
(Figs. 1–10)

Description

General.—Filarioidea, Onchocercidae, Splendidofilariinae, *Chandlerella* Yorke and Maplestone, 1926. Slender, delicate worms with slightly attenuated extremities. Buccal cavity absent, oral opening dorsoventrally elongated. Cephalic papillae consisting of four submedian pairs. Amphids in line with inner four papillae. Oesophagus long, divided externally into short, anterior, muscular part and long, posterior, glandular part. Anterior end of oesophagus often bulbous. Intestine broad. Cuticle thin, devoid of bosses or striations. Excretory pore not located and deirids apparently absent.

Male (five specimens; first figure holotype, the remainder the range of paratypes).—Length 8.6 (7.4–9.7) mm. Maximum width 95 (90–99) μ, near middle of body. Nerve ring 110 (84–111) μ from cephalic extremity.

[1]Manuscript received February 27, 1961.
Contribution from the Department of Parasitology, Ontario Research Foundation, 43 Queen's Park, Toronto 5, Ontario. This work was made possible by a research grant to the Ontario Research Foundation by the Department of Commerce and Development of the Province of Ontario.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Muscular oesophagus 116 (92–158) μ in length, dilated behind oral opening in some specimens but not in others. Glandular oesophagus 1.38 (0.44–0.56) mm in length. Total length of oesophagus 1.49 (0.61–0.71) mm. Caudal extremity tightly coiled several times. Anus 66 (57–68) μ from caudal extremity. Phasmids more or less terminal in position. Caudal papillae of holotype and two other specimens consisting of four large papillae on right side and five on left side of tail. In one specimen five papillae present on right side and four on left and in another, four on each side. Crescent-shaped swelling present immediately anterior to anus. Subequal spicules highly variable in length, arcuate, with rather pointed distal ends and differing only slightly in morphology. Lengths of spicules of five specimens respectively as follows: right 90 (holotype), 67, 106, 70, 90 μ; left 126 (holotype), 108, 67, 82, 128 μ (left spicule of third specimen shorter than right and probably abnormal).

Female (two specimens; first figure allotype).—Length 18.3, 14.2 mm. Maximum width 170, 180 μ, near middle of body. Nerve ring 158, 160 μ from anterior extremity. Muscular oesophagus 142, 153 μ in length, dilated anteriorly behind oral opening. Glandular oesophagus 613, 557 μ in length. Total length of oesophagus 755, 710 μ. Vulva 516, 350 μ (latter specimen slightly distorted anteriorly) from cephalic extremity, opening on slight swelling. Vagina muscular, directed posteriorly, approximately 330, 750 μ in length. Uteri broad, opisthodelphic, packed with microfilariae. Caudal end allotype rounded but slightly constricted short distance from caudal extremity possibly as result of presence of genital organs in tail region. In remaining specimen constriction absent and genital organs not extending to tail region. Anus in both specimens obscure and displaced to right side. Length tail 200, 150 μ. Rectum lined with thick cuticle. Phasmids not detected.

Microfilaria (10 specimens, Giemsa's stain after alcohol fixation; dead microfilariae from lung blood smeared on slide).—Medium-sized with rounded extremities, caudal end only slightly narrower than anterior. Nerve ring obscure in most specimens but represented by clear area in some. Many specimens apparently surrounded by exceedingly delicate, hyaline sheath extending a few microns beyond extremities. Inner body represented by poorly demarcated clear space. First rectal cell and excretory cell large and clear. Cephalic space present, usually containing one to three stained nuclei. Length 198 (179–208) μ. Maximum width approximately 5 μ. Positions of fixed points expressed as percentages of total length as follows: cephalic space average 4 (range 2–5) %; nerve ring 24 (21–28) %; excretory pore 35 (34–38) %; excretory cell 38

FIGS. 1–10. *Chandlerella chitwoodae* n.sp.

FIG. 1. Cephalic extremity female, lateral view (allotype).
FIG. 2. Anterior extremity female, lateral view (allotype).
FIG. 3. Microfilaria stained with Giemsa's stain.
FIG. 4. Caudal extremity male, left lateral view (holotype).
FIG. 5. Caudal extremity male, right lateral view (holotype).
FIG. 6. Caudal extremity female, lateral view (paratype).
FIG. 7. Details of anal region in preceding.
FIG. 8. Cephalic extremity female, *en face* view (paratype).
FIG. 9. Caudal extremity male, ventral view (paratype No. 4).
FIG. 10. Caudal extremity female, lateral view (allotype).

(36–40) %; anterior border of inner body 62 (61–66) %; posterior border of inner body 66 (65–68) %; first rectal cell 74 (73–76) %; anal pore 87 (86–89) %.

Host.—*Padda oryzivora* (L.) (Ploceidae).

Location.—In lungs, probably in blood vessels; microfilariae in blood.

Locality.—Java; imported to St. Thomas, Ontario, Canada.

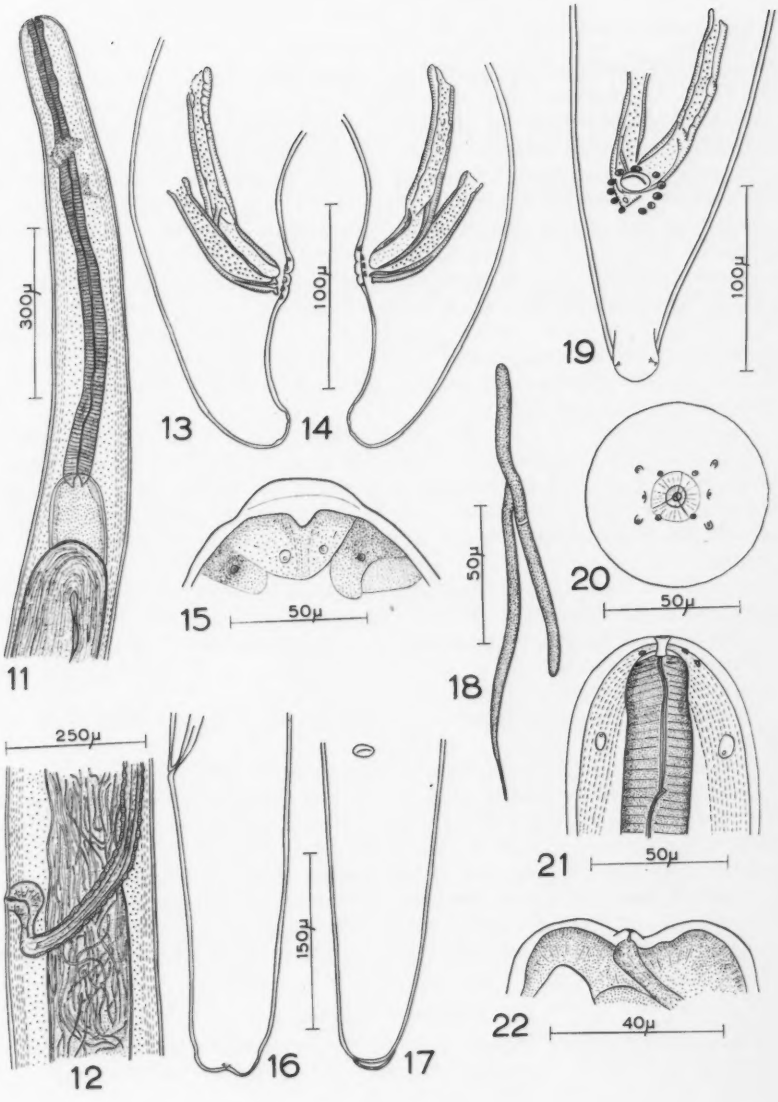
Specimens.—United States National Museum No. 59537–8 (holotype and allotype). Nematode Collection Ontario Research Foundation No. 422 (paratypes).

Comments.—*Chandlerella chitwoodae* n.sp. is similar to *Skrjabinocta petrowi* Tschertkowa, 1946 from ". . . the internal media of the eye adherent to vascular membrane" of *Streptopelia orientalis meena* (Sykes) (Columbidae) from south Kirghizia, U.S.S.R. (7) but its divided oesophagus and much longer microfilaria readily separate it from this species. Because of the great difficulty in locating the anus in the female of *chitwoodae* little importance is attached to Tschertkowa's conclusion that it is atrophied in *petrowi*. Tschertkowa, in classifying *petrowi*, was probably impressed by the site in which it was found and she compared *Skrjabinocta* to *Aprocta*. Although the arcuate form of the spicules in *petrowi* and *chitwoodae*, their morphological similarity, as well as the presence of a median, preanal, unpaired papilla are reminiscent of *Aprocta*, members of Aproctinae are oviparous and not associated with the vascular system as *petrowi* and *chitwoodae*, which produce microfilariae.[2] Anderson and Chabaud (3) tentatively referred *petrowi* to *Splendidofilaria* but later the author (1) excluded it from this genus and considered its taxonomy unsettled. Comparison of *chitwoodae* and *petrowi* with the 10 species already placed in *Chandlerella* (1) indicates they both belong in this genus. The form of the spicules of *chitwoodae* and *petrowi* might distinguish them from other *Chandlerella* but most species have not been studied in sufficient detail. The preanal, papilla-like structure has not been reported elsewhere in *Chandlerella* but more study may reveal it in other species. This structure is present in *Splendidofilaria papillocerca* (Lubimov, 1946) (5), which needs restudy as it may belong in *Chandlerella* (Spassky and Sonin (6) placed *papillocerca* in *Ornithofilaria*, which is a synonym of *Splendidofilaria* (1)). Since reliable generic characters cannot be found to separate *Skrjabinocta*

[2]*Splendidofilaria rotundicephala* (Oschmarin, 1950) was also found in the orbit of its host.

FIGS. 11–22. *Protofilaria furcata* Chandler, 1929

FIG. 11. Anterior extremity female, lateral view.
FIG. 12. Vulvar region female, lateral view.
FIG. 13. Caudal extremity male, right lateral view.
FIG. 14. Caudal extremity male, left lateral view.
FIG. 15. Body of female in oesophageal region, cross section showing thickening in cuticle.
FIG. 16. Caudal extremity female, lateral view.
FIG. 17. Caudal extremity female, ventral view.
FIG. 18. Microfilaria, unstained, from uterus of female.
FIG. 19. Caudal extremity male, ventral view.
FIG. 20. Cephalic extremity female, *en face* view.
FIG. 21. Cephalic extremity female, lateral view.
FIG. 22. Caudal extremity female, lateral view showing phasmid in cleft in tail.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and *Chandlerella* the former is considered a synonym of the latter and the combination *Chandlerella petrowi* (Tschertkowa, 1946) n. comb. is proposed.

2. *Protofilaria furcata* CHANDLER, 1929
(Figs. 11–22)

Description

General.—Filarioidea, Onchocercidae, Splendidofilariinae, *Protofilaria* Chandler, 1929 (4). Medium-sized, with tapered extremities. Buccal cavity cylindrical, thin-walled. Cephalic papillae consisting of four submedian pairs. Oral opening circular, bordered by delicate, cuticular ring. Oesophagus broad, long, undivided externally. Intestine broad, thin-walled. Cuticle thickened in lateral fields from about midway between nerve ring and cephalic extremity to region of end of oesophagus in male and vulva in female. Excretory pore not discerned and deirids apparently absent.

Male.—Length 15 mm. Maximum width 165 μ near middle of body. Oesophagus 920 μ in length. Excretory pore 270 μ from anterior extremity. Anus 95 μ from caudal extremity, surrounded by 11 rounded, flat papillae. Phasmids terminolateral. Spicules stout, markedly dissimilar in size and morphology. Left spicule 114 μ and right 79 μ in length. Caudal extremity rather irregular in outline.

Female.—Length 23 mm. Maximum width 325 μ near middle of body. Oesophagus 825 μ in length. Nerve ring 240 μ, and vulva 2.8 mm from anterior extremity. Vagina directed anteriorly where it divides into two broad uteri in region of end of oesophagus. Caudal extremity cleft, resulting in dorsal and ventral lobes between which lie phasmids. Anus 272 μ from caudal extremity.

Microfilaria (four microfilariae from preserved female specimen measured in water).—Length 250–277 μ. Width approximately 5 μ. Cuticle transversely striated and tail markedly attenuated.

Host.—*Lemur ruber.*

Location.—Thoracic cavity.

Locality.—Madagascar (specimens collected at Calcutta Zoological Gardens).

Specimens.—United States National Museum No. 8005.

Comments.—The following features of this species are reported for the first time: (*a*) the cuticle is transversely striated and thickened in the lateral fields in the anterior part of the worm; (*b*) there are four submedian pairs of cephalic papillae; (*c*) the anus of the female is not terminal as previously reported (3) and the cleft in the caudal extremity is laterolateral rather than dorsoventral; (*d*) the anus in the male is surrounded by a circle of flat papillae as noted in such genera of avian filarioids as *Paronchocerca*, *Pseudaproctella*, and *Aproctella*.

Acknowledgments

The author expresses his gratitude to Dr. Allen McIntosh and Mr. W. W. Becklund of the United States Department of Agriculture for the opportunity to study paratypes of *Protofilaria furcata*.

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[3]The author transliterates her name in her English article as Chertkova whereas in Skrjabin and Shikhobalova's monograph on the filarioids (Filariae of animals and man, Moscow, 1948) her name appears as Tschertkowa.

STUDIES ON THE LIFE CYCLE AND DEVELOPMENTAL MORPHOLOGY OF DOCHMOIDES STENOCEPHALA (RAILLIET 1884) (ANCYLOSTOMIDAE: NEMATODA)[1]

H. C. GIBBS[2]

Abstract

The life history and developmental morphology, from egg to adult, of *Dochmoides stenocephala* were investigated. Special attention is given to the development of the buccal structures, nervous system, and genitalia. Marked homology of development with that of other strongylines was observed. By the oral route, infection occurred without somatic migration. Percutaneous infection was largely unsuccessful. After infection, larvae migrated along the intestinal tract with increase in age, and the adult worms appeared to favor a location in the latter third of the small intestine.

Introduction

The nematode parasite *Dochmoides stenocephala* has been reported as occurring in dogs and other carnivores from points all across Canada and as far north as Ellesmere Island by Cameron *et al.* (4). At Fort Simpson, N.W.T., in 1956 the writer found that the incidence of this parasite in 70 dogs examined was 95%. It thus appears to be a very common parasite of our northern regions.

Although it has been known for a comparatively long time (Froelich first described it in 1789 (16)), little detailed work has been done on its biology. Fulleborn (10) studied the development of the parasitic larvae in the host tissues. Law (14) gave a brief description of the free-living stages and later Eisma (8) described the infective larvae for purposes of differentiation from those of the other hookworms.

Because of its prevalence and the need for more detailed knowledge of this parasite, studies on its biology were undertaken. A previous paper (12) discussed the effects of temperature on the development of the free-living stages. In this paper the results of studies on its life cycle and developmental morphology are reported.

Materials and Methods

Two methods were used for culturing the free-living stages. In the first, ova were collected by flotation procedures from the faeces of infected dogs and added to water cultures. These water cultures consisted of about 2 mm depth of water in small Petri dishes to which had been added a small quantity of sterile sand and a few drops of helminthologically sterile faecal suspension. The cultures were incubated at 25° C in a sloped position so as to provide an oxygen gradient, and individual cultures were removed at intervals for collection and study of the larvae. The second method was the use of charcoal-faeces cul-

[1]Manuscript received March 10, 1961.
Contribution from the Institute of Parasitology, McGill University, Macdonald College P.O., Quebec, with financial assistance from the National Research Council of Canada and Canadian Industries Limited (Wildlife Scholarship).
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tures in small Petri dishes. The larvae were recovered from the cultures by the Baermann method as required.

Infective larvae for infection experiments were obtained from faeces mixed with helminthologically sterile powdered cow manure, charcoal, and sand. These cultures were incubated at 25° C for 5 days, at the end of which time the larvae were collected in a Baermann apparatus. They were washed several times in cold tap water and stored in a refrigerator (5° C) in small volumes of water which were changed frequently until the larvae were used. Under these conditions larvae remained viable and infective after 10 months of storage. When possible 3- to 4-week-old pups that were litter mates were used for infection.

For oral administration the estimated number of larvae was concentrated in a small volume of water and administered in a gelatin capsule to the pup, which had been starved overnight. For percutaneous administration the estimated number of larvae was concentrated in a few drops of water. An area of skin in the region of the groin, which was devoid of coarse hair and where the skin was comparatively thin, was cleansed with a dry gauze pad. The infective larvae in a few drops of liquid were then applied to the skin using a Pasteur pipette. The pup was restrained in dorsal recumbency until the film of water had dried, about 20–30 minutes. The area was then sponged off with water, dried, and the pup released. Attempts at percutaneous infection were also made by injecting infective larvae subcutaneously with a hypodermic needle and syringe.

The pups were killed at specific time intervals after infection. The stomach was removed and the small intestine divided into four equal portions. The stomach and intestinal portions were then slit open with scissors, the contents washed into individual beakers, and the gastric and intestinal walls squeezed gently between the thumb and forefinger to remove adhering mucus and contents. This constituted the "washings".

The stomach and portions of the small intestine were then chopped into fine pieces with scissors and each portion digested overnight in artificial gastric juice at 37° C. The skin, lungs, liver, kidneys, spleen, and heart were also removed and examined carefully for evidence of gross lesions. Portions of these organs were then taken for histological examination and the remnant was chopped and digested overnight, and the digests examined for larvae.

The numbers of larvae were estimated by diluting the larvae in their fluid medium to a standard volume, stirring the mixture well, and removing an aliquot. The larvae present in the aliquot were then counted and the total number estimated by multiplying by the required factor. This procedure was repeated three times for each sample and the results were averaged. Portions of tissue for histological examinations were fixed in 10% formalin. The sections were stained with Ehrlich's haematoxylin and eosin.

For study, larvae were killed in hot 70% ethanol. They were then washed several times in water and concentrated by centrifugation in a small volume of water (two or three drops). An equal volume of reconstituted blood serum was added and the larvae and serum mixed by gentle rolling of the centrifuge tube. About three drops of the larvae–serum mixture were spread thinly on a slide which was then placed in a formalin cabinet and left for 24 hours to allow the serum to coagulate. Following this procedure the slides were treated like

ordinary histological slides and stained with Ehrlich's haematoxylin or Gower's alum carmine, destained, cleared, and mounted in balsam.

Adult worms were killed in hot 70% ethanol and mounted in lactophenol for study. For sectioning, adults were cleared in methyl benzoate and embedded in paraffin. There was some shrinkage but the cellular elements were clearly discernible. Sections were cut 10 μ thick and stained with haematoxylin.

The Free-living Stages

The Ovum (Fig. 2-1)

The ova of *D. stenocephala* measure 0.072–0.080 mm long and 0.045–0.055 mm wide. They are oval in shape with thin, transparent walls. When passed by the host in the faeces they are in the 8- to 16-cell stage.

Hatching of a number of larvae was observed. It began with active movements of the larva back and forth inside the shell. The anterior end of the larva was thrust against the wall until a weak spot was found, then the larva wriggled its way through the small opening formed and escaped to the outside.

The First-Stage Larva (Fig. 2-2)

The newly hatched larvae are comparatively stout and fusiform in shape, tapering slightly anteriorly and more so posteriorly. Measurements for 10 individuals are given in Table I. The greatest width occurs in the anterior portion of the body, which is fairly blunt. From the anus backwards the body tapers rapidly and the tail ends in a long narrow point. The cuticle is thin and does not appear to be striated. Muscle cells are not distinguishable but rows of elongate-muscle nuclei can be seen.

The mouth opening, which is surrounded by a small circumoral prominence, opens posteriorly into a cylindrical buccal cavity. This latter cavity has a sclerotized wall forming a ring at the posterior extremity giving the whole an inverted test-tube-like appearance. The buccal tube leads into a typically rhabditiform oesophagus. In the bulbar portion the Y-shaped valvular apparatus could be seen "winking" as the larva fed. At the end of the oesophagus two pairs of cells, the future oesophageal valves, can be seen.

The intestine is composed of eight dorsal and eight ventral cells, the nuclei of which alternate with each other, causing the intestinal lumen to present a

TABLE I

Measurements (mm) of free-living larval stages

Item	Range	Mean	Range	Mean	Range	Mean
Stage	I		II		III	
Total length	0.29–0.36	0.32	0.43–0.53	0.48	0.50–0.58	0.53
Maximum width	0.017–0.021	0.018	0.023–0.027	0.025	0.018–0.023	0.021
Length of oesophagus	0.081–0.100	0.094	0.11–0.13	0.12	0.15–0.17	0.16
Nerve ring from ant. end	0.060–0.070	0.064	0.072–0.087	0.077	0.075–0.100	0.085
Excretory pore from ant. end	0.063–0.081	0.069	0.073–0.093	0.084	0.080–0.110	0.089
Genital primordium from ant. end	0.15–0.22	0.18	0.26–0.30	0.27	0.28–0.34	0.30
Length of tail	0.050–0.060	0.056	0.066–0.075	0.073	0.065–0.075	0.070
Number measured	10		10		10	

wavy appearance. The beginning and end of the lumen appears to be wider than the rest of it. The cells contain a number of granules and these increase with age.

The rectum is a fine, slit-like, sclerotized canal directed caudoventrally. At the rectointestinal junction the three large cells of the rectal ligament can be seen.

The excretory pore is situated just posterior to the nerve ring on the ventral side. It is continued posteromedially by a very narrow canal which is obscured by the nuclei crowded in this area.

The nervous system consists of the circumoesophageal ring situated at about the mid-point of the oesophageal isthmus. Immediately anterior to this is a group of nuclei which represent the rudimentary ganglia of the cephalic papillary nerves. The latter are represented by chains of nuclei extending anteriorly from the rudimentary papillary ganglia. Posterior and dorsal to the nerve ring there is a small group of cells which represent the rudimentary dorsal and subdorsal ganglia. Ventral to the nerve ring a number of nuclei are distinguishable from which the subventral and ventral ganglia develop. Continuing ventrally and posteriorly, at the level of the oesophagointestinal valve, the primordium of the retrovesicular ganglion is seen. The primordial cells of the lateral ganglia may also be distinguished lying just posterior to the nerve ring on the lateral aspect.

The genital primordium lies just opposite the junction of the fourth and fifth intestinal cells. It is composed of four cells arranged typically in a row forming a small oval body (Fig. 5-B(1), B(11)). At the apices are the two epithelial cells, which are crescent-shaped, and in the center are two germinal cells whose nuclei are more granular and less intensely stained by haematoxylin. It was not possible to differentiate the sexes at this stage.

Four coelomocytes are present. There is one associated with the genital rudiment and the other three are situated approximately equidistant from each other and from the genital coelomocyte in the area between the genital primordium and the oesophagointestinal junction.

The Second-Stage Larva (Fig. 2-3)

The second-stage larva is similar in shape to the first-stage larva but is less blunt anteriorly and much longer. Measurements of 10 individuals are given in Table I. The cuticle is striated unlike that of the first stage.

The organization of this larva is very similar to that of the preceding stage.

The intestinal cells increase in number so that there are 16 present.

The genital primordium shows an increase in the number of epithelial cells so that by the end of the second stage there are eight epithelial cells and two germinal cells. In this stage as well the sexes were not distinguishable (Fig. 5-B(2), B(12)).

The Free-living Third-Stage Larva (Fig. 2-4)

The third-stage larva is ensheathed in the cuticle of the previous stage and appears to be more slender than the second-stage larva. The measurements of 10 individuals are given in Table I.

The cephalic extremity bears two subventral papillae, two subdorsal papillae, and two lateral amphids.

Both the cuticular sheath and the larval cuticle are marked by fine transverse striations.

The cells of the somatic musculature may be distinguished by their small, elongate nuclei, which occur in rows in the four longitudinal sectors of the body.

This larva does not feed and hence the mouth is much reduced from the previous stage. It leads into a narrow lumen. The oesophagus is long and slender with a relatively inconspicuous bulb. The intestinal cells, which number 32, can be distinguished by their large oval nuclei.

The excretory pore, which opens just posterior to the nerve ring, is continued by means of a fine canal whose course is obscured by the mass of nuclei posterior to the nerve ring. The primordia of the excretory glands are visible as large, fusiform bodies with sausage-shaped nuclei lying between the intestine and the body wall, a little anterior to the genital primordium.

Immediately anterior to the nerve ring the primordia of the cephalic papillary ganglia are readily seen. From these the six rudimentary papillary nerves may be seen proceeding anteriorly as fine rows of nuclei. The lateral nerves are amphidial nerves; the four sublateral nerves are papillary nerves. The nerve ring encircles the oesophagus at about its middle. Just posterior to it on the dorsal aspect the two subdorsal ganglia and the single dorsal ganglion are seen as three clusters of nuclei; the paired subdorsal group situated one on each side of the dorsal group. Lateral to these and extending caudad are the large paired groups of the lateral ganglia, which are continued into the postlateral ganglia as far as the oesophageal bulb (Fig. 2-6(B), 6(C)).

From the ventral and lateral aspects can be seen three groups of nuclei extending in sequence from the nerve ring. These are the large paired ventral ganglia, a group surrounding the excretory duct, the posteroventral ganglion, and finally, just anterior to the oesophageal bulb, the retrovesicular ganglion. From this latter group a long chain of nuclei extends posteroventrally, eventually entering a group of nuclei, the anal or preanal ganglion, just anterior and ventral to the rectum. The paired lumbar ganglia lie dorsal, lateral, and just posterior to the anal ganglion and from these proceed the primordia of the phasmidial nerves, which lead to the paired phasmids one on each side of the tail (Fig. 2-6(A)).

At this stage the genital rudiments show definite sexual dimorphism. The male rudiment starts to bulge at the anterior end so that it appears comma-shaped. The two genital cells are situated close together at the posterior pole of the rudiment and the epithelial cells number about 8 to 10 (Fig. 5-B(4), B(5)). The female rudiment at this stage is oval. The genital cells are situated one at each pole of the rudiment and the epithelial cells number about 12 (Fig. 5-B(13)).

The coelomocytes are still distinguishable situated as in previous stages.

Infection

Oral Infection

Oral infection appeared to result in development without the need for somatic migration. The results are summarized in Table V and Fig. 1. While there was good recovery of worms from the alimentary tract, none were obtained from the viscera.

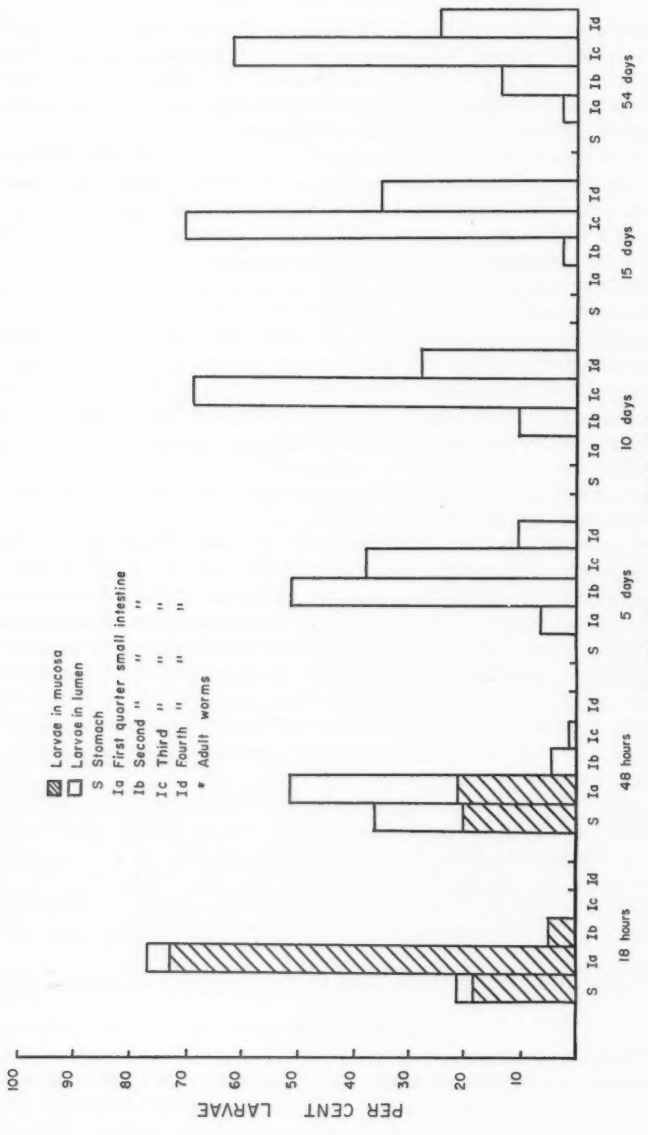


FIG. 1. Migrations of larvae in the gastrointestinal tract with increase in age.

On entering the stomach, the larvae shed their sheaths. Some burrowed deeply into the lumen of the mucous glands of the stomach in the pyloric region but most made their way to the duodenum where they, too, entered the lumen of the intestinal glands and became coiled. Of the larvae recovered from a pup 18 hours after oral infection, 19.2% were found in the gastric mucosa, 70.5% in the duodenal mucosa, and 4.2% in the ileal mucosa. In addition some larvae were still free in the lumen of the stomach (2%) and in the first quarter of the small intestine (4%).

Forty-eight hours after infection most of the larvae appeared to have completed their stay in the mucosal glands and had emerged into the gastric and intestinal mucosa. All larvae free in the lumen were equipped with a provisional buccal capsule and were in lethargus or undergoing ecdysis.

Five days after infection the greatest concentration of larvae was found in the second quarter of the small intestine.

These were all in the fourth stage. At about 7 to 8 days the fourth ecdysis occurred and by 10 days all the larvae had reached the fifth stage. At 15 days the adults were mature and ova were present in the host's faeces.

Percutaneous Infection

The results of a series of experiments to see whether infection could be established by the percutaneous route were almost negative. Very few larvae were able to set up infection by this route.

Eighteen hours after infection a few larvae had reached the lungs and were in the parasitic third stage. Examination of other viscera microscopically, by digestion, and histological section, gave negative results.

Four days after applying 3000 infective larvae to the skin, 10 larvae were found in the lumen of the first and second quarters of the small intestine. From this point on, development paralleled that seen when larvae were given orally.

When infective larvae were given subcutaneously using a needle and syringe the results were negative. Two days after inoculation larvae were recovered from the region of inoculation by digestion procedures but none were found in the lungs, other viscera, or intestinal tract.

The Parasitic Stages

The Parasitic Third Stage

This stage externally is very similar to the previous stage except that its sheath is absent, but, in addition, some changes are observed in internal organization.

The musculature is essentially the same as in the previous stage and is seen as rows of about 30 to 36 elongated nuclei in the four sublateral sectors of the body.

Initially the mouth is much reduced and consists of a narrow tube leading into a cavity with thick walls. As these larvae become older, however, the beginning of the provisional buccal capsule is seen. The first sign of this is the development of a pair of vacuolations in the dorsal and ventral fields at the anterior end of the oesophagus (Fig. 6-1). These unite laterally, the walls become sclerotized, and the developing buccal capsule is then clearly seen as a goblet-shaped structure. A feeding tube still remains in the middle, connecting

the oesophagus with the exterior. At the base of the capsule two pairs of lancets develop (Fig. 6-2, 3). At the time of the third lethargus the provisional buccal capsule is seen as a dorsally directed, cup-shaped structure, with the remains of the feeding tube still present (Fig 6-4).

The oesophagus is long and slender with a slight bulb at its posterior end. The intestinal cells number 32 and their nuclei are large and oval.

The excretory canal is hard to distinguish as the fine canal is obscured by the mass of nuclei in the region of the nerve commissure. The primordia of the cervical glands have elongated considerably and are prominent on either side of the intestine.

There is little change in the genital rudiment in either sex during this stage (Fig. 5-B(6), B(7)). In the male there is an increase in cells in the anal region and the beginning of the spicular primordia are in evidence in older larvae.

Fourth-Stage Larvae (48 hours) (Fig. 2-5, Fig. 3)

These larvae are characterized by the presence of a provisional buccal capsule, which is goblet-shaped with the aperture directed anteriodorsally. It is about 0.018 mm deep. The opening is circular and not guarded by plates (Fig. 6-5, 6). Measurements of 10 individuals are given in Table II.

TABLE II

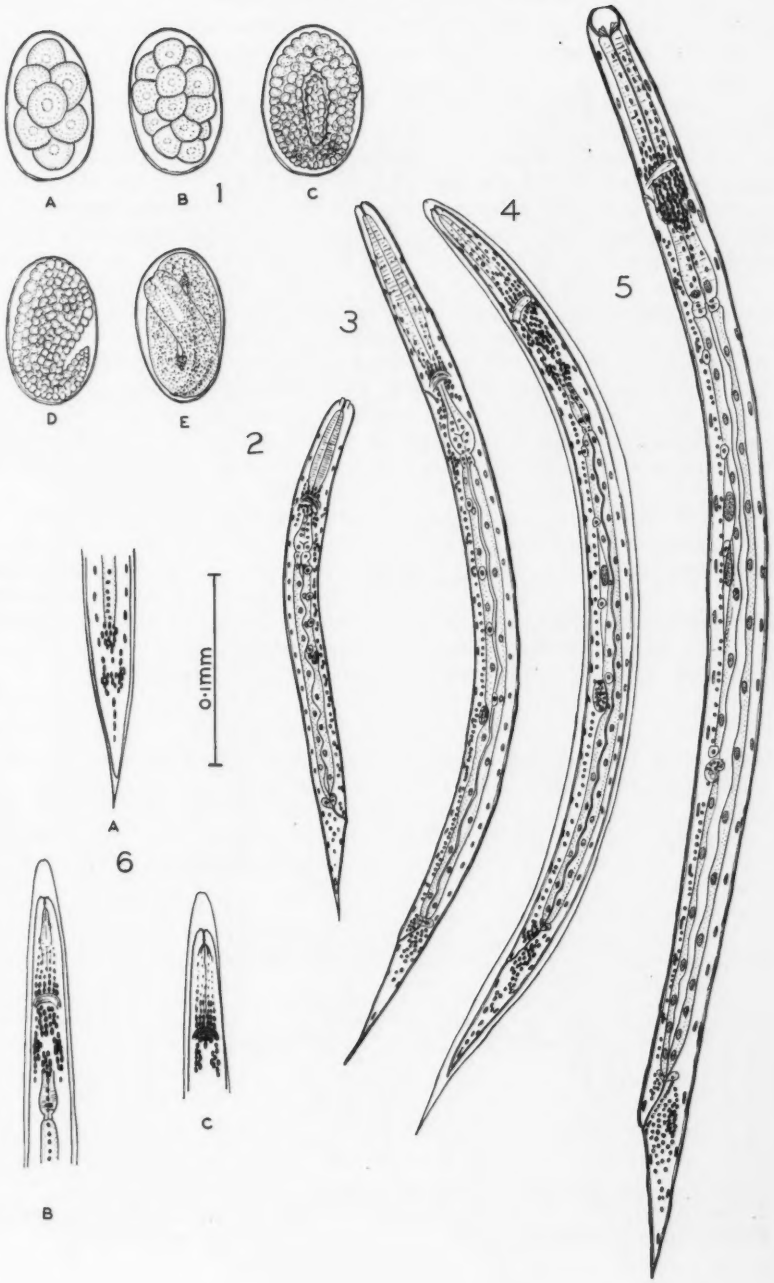
Measurements (mm) of fourth-stage larvae (48 hours)

Item	Male		Female	
	Range	Mean	Range	Mean
Total length	0.718–0.823	0.772	0.753–0.875	0.781
Maximum width	0.023–0.028	0.027	0.028–0.032	0.029
Nerve ring from ant. end	0.105–0.116	0.112	0.105–0.123	0.109
Ex. pore from ant. end	0.105–0.116	0.112	0.105–0.123	0.109
Length of oesophagus	0.193–0.210	0.196	0.193–0.210	0.196
Genital primordium from ant. end	0.385–0.543	0.487	0.508–0.630	0.543
Length of tail	0.053–0.070	0.068	0.063–0.070	0.069
	10		10	

At the base of the oesophagus in each sector a large nucleus may be seen corresponding to each of the three oesophageal glands. At the junction of the oesophagus and intestine the nuclei of the oesophageal valves are distinguishable. The intestine consists of 32 cells with large oval nuclei. It leads into the rectum, a sclerotized tube guarded at its origin by the intestinorectal sphincter, and the cells of the rectal ligament.

The bodies and nuclei of the cervical glands are readily distinguishable. They extend by means of delicate necks from the vicinity of the excretory pore just

FIG. 2. 1. Development of ovum: (A) 8-celled stage; (B) 16-celled stage; (C) gastrula; (D) tadpole; (E) embryonated.
2. First-stage larva, lateral view.
3. Second-stage larva, lateral view.
4. Third-stage preparasitic larva, lateral view.
5. Fourth-stage (48 hours) male larva, lateral view.
6. Details of nervous system, preparasitic third-stage larva: (A) ventral view of tail; (B) ventral view of anterior end; (C) dorsal view of anterior end.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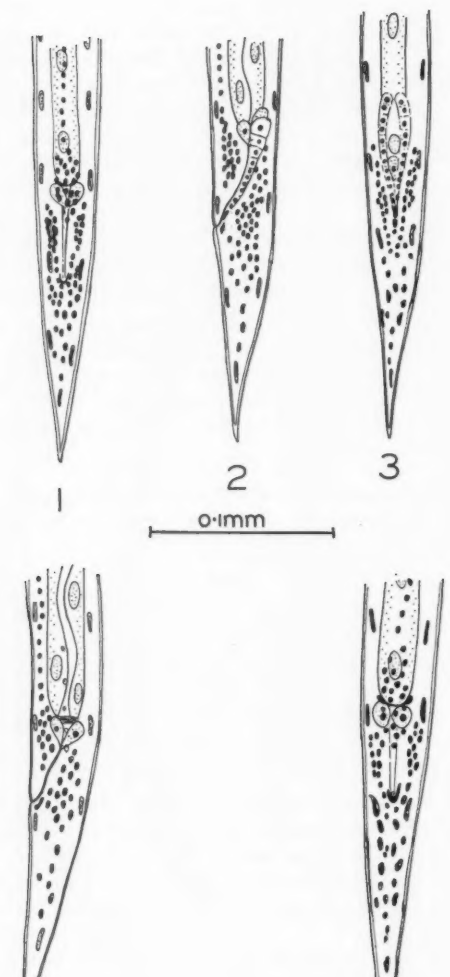


FIG. 3. Details of tail of fourth-stage larva (48 hours):
1. Ventral view, male. 2. Lateral view, male. 3. Dorsal view, male. 4. Lateral view, female. 5. Ventral view, female.

anterior to the genital rudiment. The nuclei of the cephalic glands lie just posterior and dorsal to the nerve ring but the body of the gland is less easily seen.

In the region of the nerve ring the nuclei of the ganglial primordia have become aggregated into well-defined groups. The lateral ganglia appear as pendulous clusters, while the single dorsal and paired subdorsal ganglia are smaller. The subventral and retrovesicular ganglia are as in the third stage. The anal ganglion is seen posteriorly and ventrally in the region of the intestinorectal junction. Dorsolateral to the rectum may be seen the lumbar ganglia. The ventral nerve is readily seen as are the beginnings of the lateral nerves.

The sexes are easily separable at this stage. The genital rudiment in the male is comma-shaped and there is a gradual rotation of the rudiment so that the germinal cells, which were at the posterior pole, come to lie at the anterior pole. This rotation appears to be complete after 3 days in the host (Fig. 5-B(8), B(9), B(10)). In the female the rudiment is flattened and oval and consists of about 16–18 epithelial cells. The two germinal cells are at opposite ends and are capped by polar epithelial cells (Fig. 5-B(15), B(16)). There is no readily apparent connection of the female rudiment to the body wall.

In the tail region the male larva shows a large number of nuclei. The nuclei of the cells which comprise the spicular primordia are distinguishable as two rows dorsal to the cloaca. Other cells present give rise to the muscles which control the spicules and cloaca (Fig. 4-2, 3).

In both sexes in the region of the intestinorectal sphincter may be seen the three cells of the rectal ligament, one dorsal and two lateroventral (Fig. 3-1, 2, 4, 5). In addition a semilunar nucleus was observed just posterior to the anus (Fig. 3-1, 5).

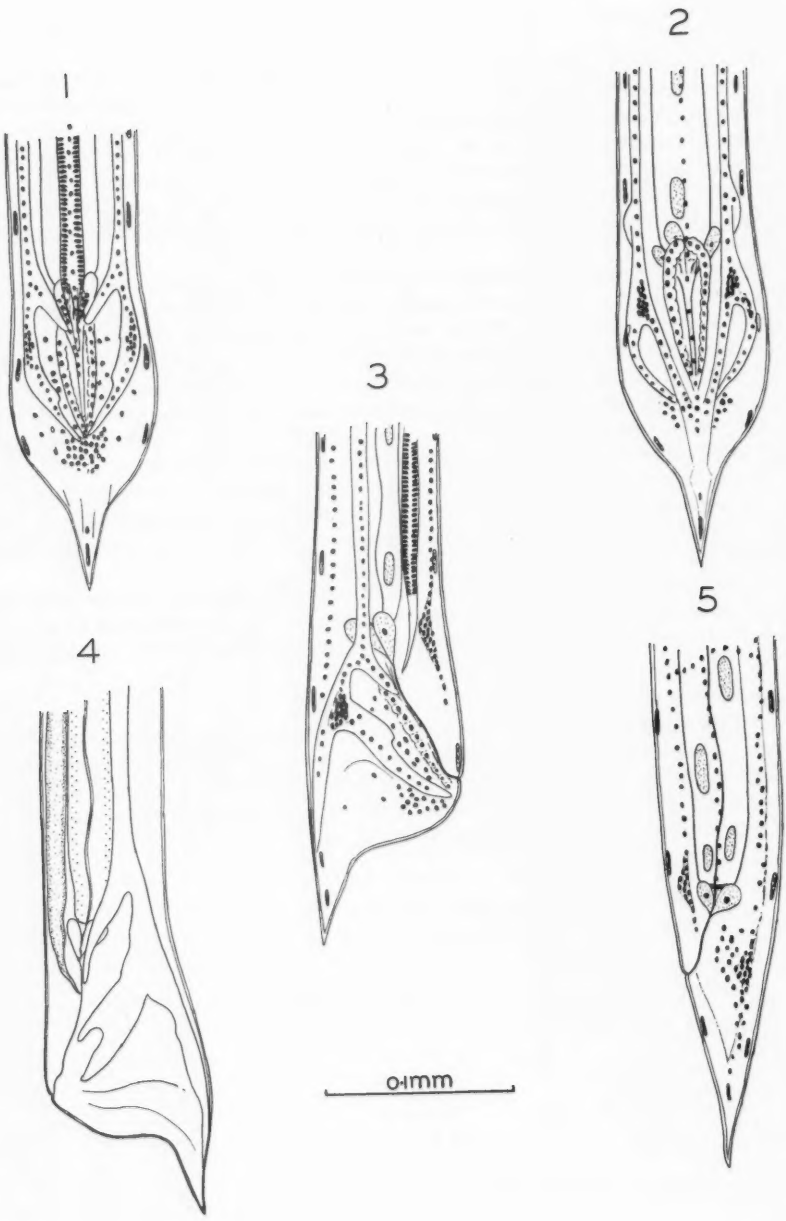
Fourth-Stage Larvae (5 days) (Fig. 4)

At this stage the larvae have increased greatly in size from the previous stage. Measurements of 10 individuals are given in Table III. Sexual dimorphism is evident at a glance. The female is provided with a pointed tail (Fig. 4-5), while the male shows a bulbous swelling in the anal region, which is terminated by a sharp point (Fig. 4-1, 2, 3, 4).

TABLE III

Measurements (mm) of fourth-stage larvae (5 days)

Item	Male		Female	
	Range	Mean	Range	Mean
Total length	1.90–2.28	2.15	2.10–2.56	2.43
Maximum width	0.07–0.09	0.08	0.08–0.09	0.08
Nerve ring from ant. end	0.20–0.22	0.21	0.21–0.26	0.23
Ex. pore from ant. end	0.24–0.27	0.26	0.25–0.29	0.27
Cervical papilla from ant. end	0.25–0.29	0.27	0.27–0.29	0.28
Length of oesophagus	0.38–0.42	0.41	0.43–0.47	0.45
Vulva from ant. end	—	—	1.56–1.70	1.65
Length of tail	—	—	0.12–0.14	0.13
Number measured	10		10	


1
2
3
4
5
0·1mm

Development of the buccal capsule is initiated as a vacuolation around the base of the provisional buccal capsule. The walls of this vacuolation become sclerotized as it increases in size. By the late fourth stage the buccal capsule is seen below the provisional buccal capsule and the latter is connected by means of a fine tube to the oesophagus. It is shed at the final molt (Fig. 6-7, 8, 9, 10).

The nerve trunks, lateral, ventral, and dorsal, are discernible as longitudinal rows of nuclei. At intervals along these trunks may be seen secondary commissures or lateral branches consisting of groups of a few nuclei. At this stage indications of the development of the bursa in the male can be seen under the cuticle, dorsolateral to the rectum. The rays of the bursa originate from outgrowths of the lateral nerves. At about the level of the intestinorectal sphincter a short branch arises which gives rise to the ventral rays. The main branch then splits into two, one of which proceeds posterodorsally to join with the dorsal nerve trunk and give rise to the dorsal rays. The other proceeds posteroventrally and gives rise to the lateral bursal rays. The spicules and spicular sheath appear to originate from a group of primordial cells which lie in two rows dorsal to the cloaca (Fig. 4-1, 2, 3, 4).

The genital primordia show marked differences and resemble the adult form. In the male the numerous cells of the rudimentary testes may be seen at the anterior end of the rudiment. Following this is a row of about three cells which comprise the rudimentary seminal vesicles. Next, a shorter, denser portion considered to be the *vas efferens*, which leads into a less dense row of cells, and finally into the *vas deferens* and cloaca (Fig. 5-C). The male genitalia in the early fifth stage is shown in Fig. 5-A. In the female the genital rudiment is seen to have proliferated in an anteroposterior direction (Fig. 5-D). At the middle is a portion consisting of a double row of cells, while at the poles the cells are in a single row with the two germinal cells at either end capped by polar epithelial cells. At this point the rudiment is attached to the body wall by a number of elongated cells which represent the rudimentary vagina and vulva. As the larval female grows older the double row of cells in the center portion of the rudiment forms the walls of the ovejectors. The polar rows of cells increase in number and may be differentiated into two major portions, the portions adjacent to the primitive ovejectors which give rise to the uteri, and the terminal portions which become reflected back over the uteri to form the ovaries (Fig. 5-E).

Fifth Stage or Adult (Figs. 7, 8)

External Appearance

The adult worms are a pink color when removed from the host. The measurements of 10 individuals for both sexes are given in Table IV.

FIG. 4. Details of tail of fourth-stage larva (5 days):
1. Male tail, ventral view.
2. Male tail, dorsal view.
3. Male tail, lateral view.
4. Male tail, lateral view, showing developing bursa.
5. Female tail, lateral view.

An *en face* view of the adult (Fig. 5-6) shows two lateral amphids as well as two dorsolateral and two latroventral papillae. No internal cephalic papillae are present. A pair of lateral cervical papillae is seen just posterior to the level of the nerve ring.

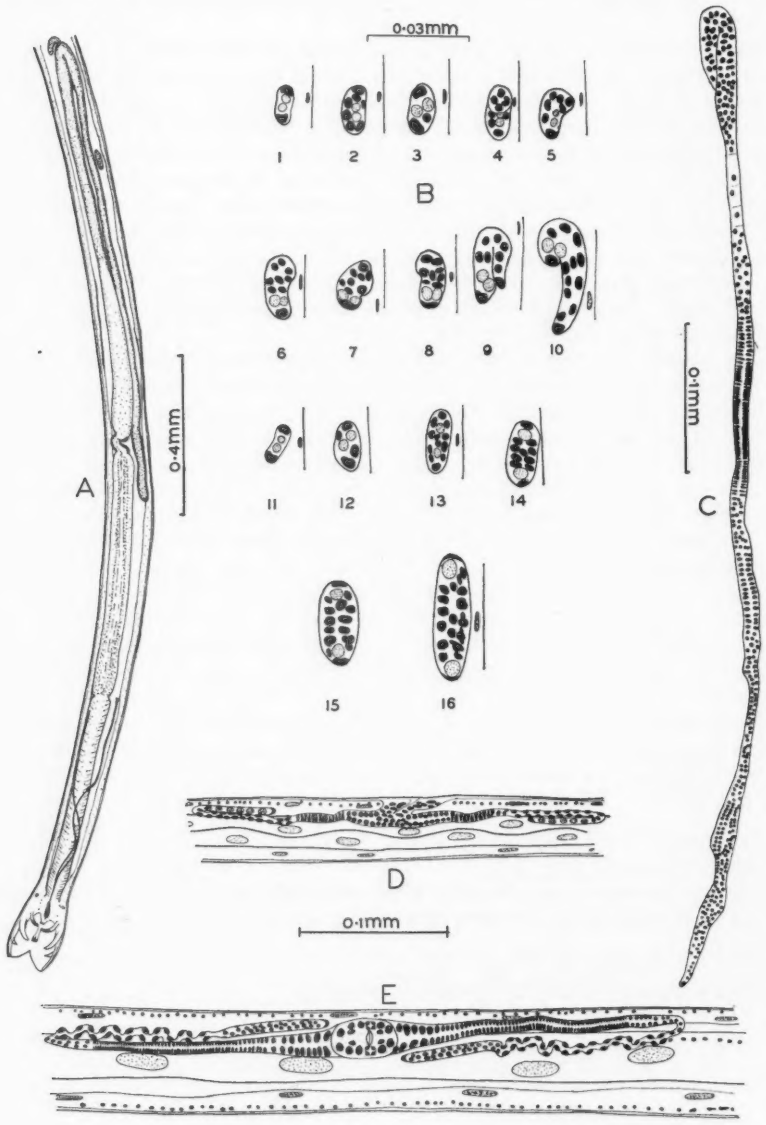


TABLE IV

Measurements (mm) of adults

Item	Male		Female	
	Range	Mean	Range	Mean
Total length	7.95 –9.38	8.77	10.43–12.54	11.30
Maximum width	0.195–0.210	0.202	0.210–0.250	0.231
Nerve ring from ant. end	0.450–0.480	0.462	0.525–0.540	0.533
Ex. pore from ant. end	0.480–0.525	0.513	0.525–0.600	0.560
Cervical papilla from ant. end	0.510–0.555	0.534	0.555–0.630	0.591
Length oesophagus	0.855–0.915	0.914	0.990–1.095	1.023
Vulva from ant. end	—	—	6.525–8.320	7.222
Length spicules	0.675–0.750	0.711	—	—
Length of gubernaculum	0.090–0.105	0.099	—	—
Length of tail	—	—	0.180–0.255	0.209
Number measured	10		10	

The cuticle in both sexes shows fine transverse striations.

The bursa in the male resembles that of *Ancylostoma* (15) (Fig. 7-4, 5; Fig. 8-9). There is a prominent pair of ventral prebursal papillae. The bursa tends to remain closed in fixed specimens and consists of a short dorsal lobe and two large semicircular lateral lobes. The dorsal ray is bifurcated near its tip, each branch being tridigitate, of which the outermost is the longest. The rays tend to be thicker than those of *Ancylostoma*. The genital cone is fairly prominent and bears on its apex four cone-shaped protuberances.

The tail of the female is slightly compressed dorsoventrally, just posterior to the anus. It is adorned with a small terminal spike (Fig. 7-1).

Body Wall

The cuticle is about 0.013 mm thick. It consists of two layers, cuticle and subcuticle, both of which are finely striated, as described by Looss (15) for *Ancylostoma* and Cameron (2) for *Monodontus*. It is very thin over the buccal capsule.

The longitudinal bands are very similar to those described by Looss (15) for *Ancylostoma*.

The cephalic glands are hard to discern except for the nucleus, which, in stained specimens, is seen just posterior to the level of the nerve ring. The musculature of the body wall is platymyarian and meromyarian. All of the muscles appear to give off sarcoplasmic processes (Fig. 8-1, 2, 3).

In the cephalic region four pairs of dorsal muscles may be seen. In addition there are muscles in connection with the male and female genitalia, anus, and cloaca.

FIG. 5. Development of the genitalia: A. Male, subadult. B. Development of genital rudiment: (1) male, first-stage larva; (2) male, second-stage larva; (3) male, second-stage larva; (4) male, third-stage larva; (5) male, third-stage larva; (6) male, parasitic third-stage larva; (7) male, parasitic third-stage larva; (8) male, early fourth-stage larva; (9) male, early fourth-stage larva; (10) male, early fourth-stage larva; (11) female, first-stage larva; (12) female, second-stage larva; (13) female, third-stage larva; (14) female, parasitic third-stage larva; (15) female, early fourth-stage (48 hours) larva; (16) female, early fourth-stage (48 hours) larva. C. Male, late fourth-stage (5 days) larva. D. Female, late fourth-stage (5 days) larva. E. Female, subadult.

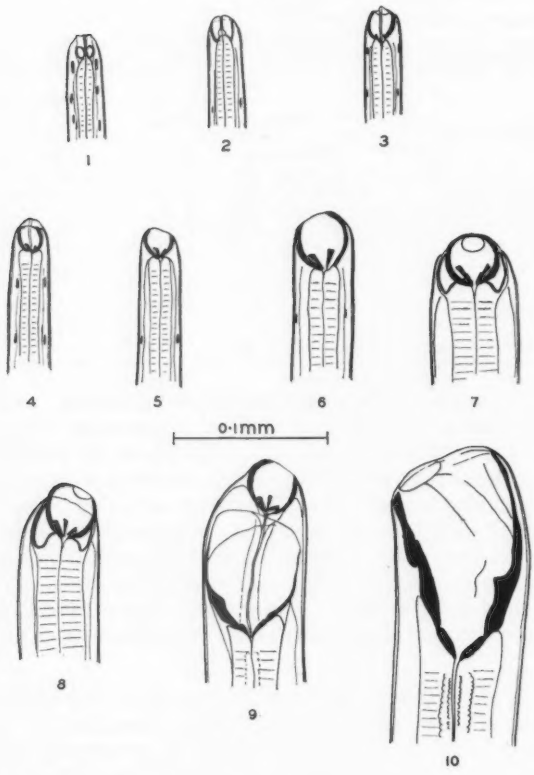


FIG. 6. Development of buccal capsule:
1. Parasitic third stage, beginning of provisional buccal capsule.
2. Parasitic third stage, later stage than 1.
3. Parasitic third stage, provisional buccal capsule formed.
4. Early fourth stage, undergoing ecdysis.
5. Early fourth stage.
6. Fourth-stage larva, full-sized provisional buccal capsule.
7. Late fourth stage, beginning of permanent buccal capsule, ventral view.
8. Lateral view of same larva as in 7.
9. Completion of development of permanent buccal capsule.
10. Lateral view of buccal capsule in subadults.

Digestive System

The buccal capsule is cone-shaped (Fig. 7-2; Fig. 8-10). Its walls are heavily sclerotized and are thickest at the base of the capsule. The oral aperture, which is circular, is protected by two ventral semilunar cutting plates. Smaller non-cutting plates are found at the dorsal edge of the opening. The average depth of this structure is 0.164 mm in the male and 0.189 mm in the female. On the ventral inner wall of the capsule project two cutting lancets. There is no dorsal oesophageal cone, the salivary gland opening as in *Ancylostoma* on the dorsal

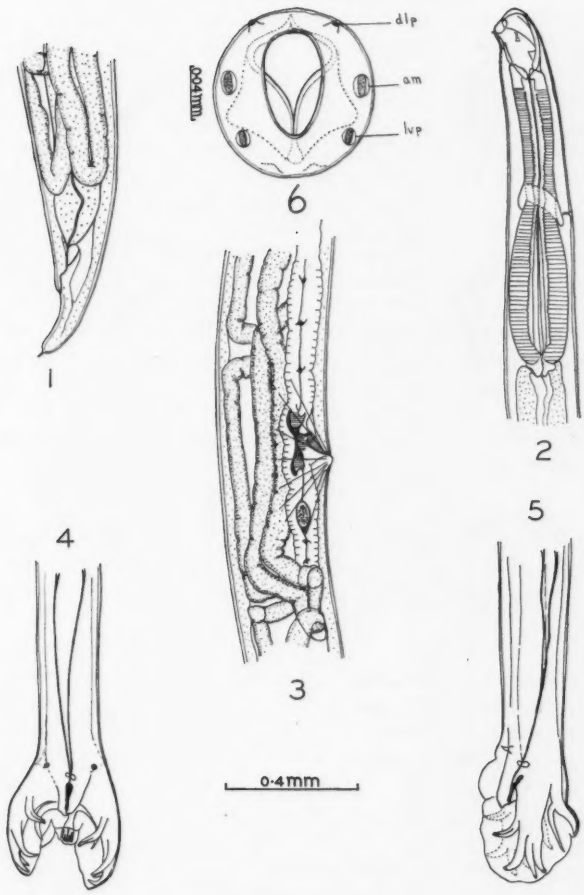


FIG. 7. Adult: 1. Female tail, lateral view.
2. Male, lateral view of anterior extremity.
3. Female, lateral view of detail of ovejector.
4. Male tail, ventral view.
5. Male tail, lateral view.
6. Anterior extremity, *en face* view; *am* amphid, *dlp* dorsolateral papilla, *lvp* lateroventral papilla.

wall of the capsule. A cephalo-oesophageal ligament connects the mouth capsule to the oesophagus.

The oesophagus averages 0.750 mm long in the male and 0.834 mm long in the female. It consists of an anterior neck and a posterior bulbar portion. It has a triradiate sclerotized lining. At its anterior end it forms a funnel-shaped structure at the base of the buccal capsule. At the posterior end the entrance

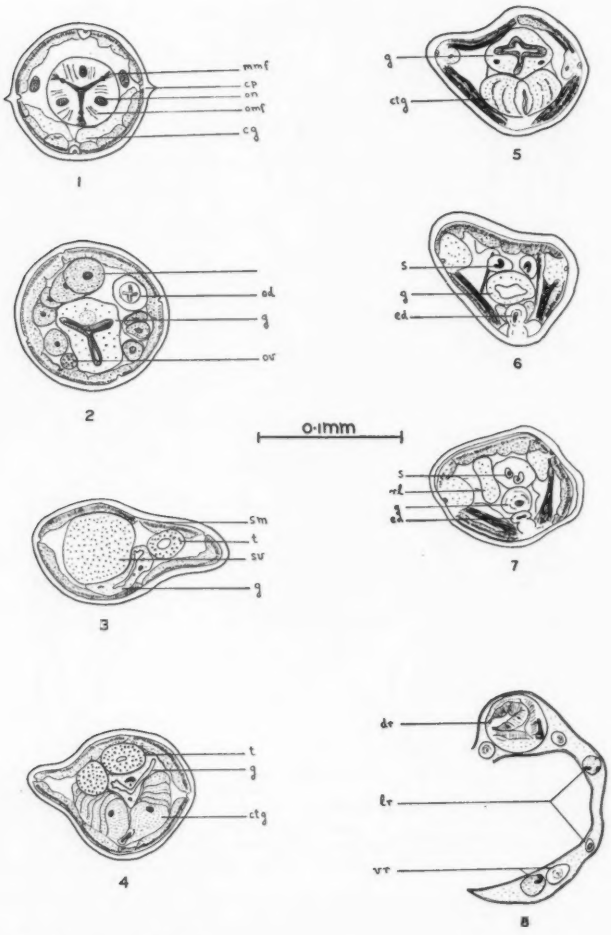


FIG. 8. Cross sections of adult:
1. Female, in region of cervical papillae.
2. Female, anterior to middle of body.
3. Male, through seminal vesicle.
4. Male, below seminal vesicle.
5. Male, below 4.
6. Male, at level of spicular origin.
7. Male, just anterior to cloaca.
8. Male, through bursa.

ABBREVIATIONS: *cg* cephalic gland, *cp* cervical papilla, *ctg* cement gland, *dr* dorsal ray *ed* ejaculatory duct, *g* gut, *lr* lateral rays, *mmf* marginal muscle fibers, *o* ovum, *omf* oesophageal muscle fibers, *on* oesophageal nucleus, *ov* ovary, *rl* rectal ligament, *s* spicules *sm* somatic muscle, *sv* seminal vesicle, *t* testis, *vr* ventral rays.

to the gut is guarded by three flaps which act as valves. Three longitudinal nerve fibers are found in the substance of the oesophagus and in addition there are three glands, one dorsal and two lateroventral. The dorsal gland empties on the dorsal aspect of the buccal capsule. The others empty just anterior to the nerve ring. The nuclei of these glands lie at the posterior end of the oesophagus. The musculature of the oesophagus is of two different kinds, marginal and ordinary. The former runs from the three radii of the lumen, while the latter makes up the bulk of the oesophageal mass.

The intestine consists of two rows of cells which alternate with each other causing the lumen of the intestine to have a wavy appearance. In the sexually immature fifth-stage worm the nuclei of the cells are seen as large oval bodies, one nucleus to each cell with a total of 32. In the sexually mature fifth stage, however, on cross section the intestinal cells are seen to be polynucleate, the nuclei are seen as numerous small basophilic structures (Fig. 8-3). The lumen of the gut is lined by a thick, compact bacillary layer resting on a subbacillary layer. The cells often contain a dark brownish pigment. This was considered by Looss (15) in the case of *Ancylostoma* to be derived from blood. The intestine terminates in the rectum. At the intestinorectal junction there is a horseshoe-shaped sphincter muscle. At this point also is seen the large cellular mass known as the rectal ligament.

The rectum in the female is a cuticularized flattened tube about 0.1 mm to 0.2 mm long which empties to the outside by means of a simple anus. In the male it is similar but receives the ejaculatory duct and spicules and forms the cloaca.

Excretory System

The excretory apparatus consists of the paired cervical glands which extend from the region of the excretory pore to about the middle of the body. The large, dark-staining nuclei may be seen in prepared specimens. In all respects they appear identical with those of *Ancylostoma*.

Nervous System

The nerve ring is situated in the anterior portion of the body and surrounds the neck of the oesophagus. Associated with it are five main ganglia, two lateral, two ventral, and a single dorsal ganglion. These are better seen in the immature and larval stages of the worm. Two lateral, a dorsal, and a ventral nerve may also be distinguished in stained specimens.

At the posterior extremity is seen a large ventral preanal ganglion, paired lumber ganglia, and an anorectal commissure. The two ventrolateral commissures as figured by Looss (15) were not discernible. The innervation of the bursa is similar to that for *Ancylostoma*.

Genital Organs

The male genital organs are very similar to those of *Ancylostoma*. The testicular coils do not surround the intestine in a helical fashion but zigzag back and forth in loops on either side of it. The testis is continued into a spindle-shaped seminal vesicle. The seminal vesicle is about 1.00 mm long and on cross section is seen to be filled with spermatozoa. The cement gland joins the seminal vesicle by means of a short tube. The cement gland in cross section at its anterior end is grooved on its dorsal surface giving each side the appearance

of a wing with the ejaculatory duct running through the center of it (Fig. 8-4, 5, 6, 7). Towards the posterior end of the worm it becomes more circular in cross section. It is seen to consist of layers or rows of flattened cells. The spicules average 0.711 mm in length and consist of round, filiform rods with sharp points which taper slightly from their proximal end. They are borne in cuticularized sheaths dorsal to the intestine. Their proximal ends are oval in outline, open, and receive the spicular pulp (Fig. 8-6, 7, Fig. 7-4, 5). On the dorsal wall of the cloaca is seen the oval, trough-shaped gubernaculum. This is about 0.099 mm long. The genital cone is fairly prominent, it bears the cloacal opening subterminally on its dorsal side. The apex is ornamented by four cone-shaped cuticular prominences.

The female genitalia are similar to those of *Ancylostoma*. The ovaries commence in the anterior end of the worm near the end of the oesophagus and run together until near the vulva. The posterior ovary continues to near the anus, turns, and joins the uterus. The uteri tend to lie longitudinally rather than in zigzag fashion as in *Ancylostoma*. At its termination the uterus leads into the ovejector (Fig. 8-3). This consists, as in *Ancylostoma*, of two parts, the longer *pars haustrix*, connected to the uterus, which leads into a short vagina, which in turn opens on a vulvar prominence on the ventral surface of the body by means of a transverse slit. A number of diagonal muscle fibers may be seen connected to the ovejector. The ovejector itself consists of a single layer of muscle tissue. Four valvular sections can be distinguished in each *pars haustrix*. At the junction of the ovary and the uterus a portion containing sperm, the seminal receptacle, can be seen.

TABLE V

Numbers of larvae recovered after oral infection*

Duration of infection Larvae given (approx.)	18 hr 4000	48 hr 1000	5 days 5000	10 days 8000	15 days 1500	54 days 2000
Stomach washings	35	108	0	0	0	0
Digest	300	140	0	0	0	0
Small intestine						
First quarter						
Washings	60	275	320	0	0	0
Digest	1100	144	0	0	0	0
Second quarter						
Washings	0	25	2900	20	20	237
Digest	65	0	0	0	0	0
Third quarter						
Washings	0	5	2000	6000	600	1200
Digest	0	0	0	0	0	0
Fourth quarter						
Washings	0	0	400	2750	300	440
Digest	0	0	0	0	0	0

*Digests of lungs, liver, kidney, spleen, and heart were negative for larvae.

Discussion

The life cycle and developmental anatomy of *D. stenocephala* is typical of that described for other ancylostomes. Development of the free-living stages has been shown to be influenced by the environmental temperature by Gibbs and Gibbs (12). This worm appears to be an opportunist insofar as temper-

ature is concerned, taking full advantage of upward shifts in environmental temperature to further attainment of the infective stage, and appears to be adapted to survival in a northern environment. Marked developmental homology with other strongyles that have been studied was noted. Thus the development of the nervous system closely parallels that described by Looss (15) (16) for *Ancylostoma*, Alicata (1) for *Hyostrongylus*, and Schad (21) for *Kalicephalus*. This is also true of the buccal capsule and that of *Monodontus* (3), *Ancylostoma* (26), and *Gaigeria* (18). Close parallelism in development of the male bursa is seen with that of *Gaigeria* and *Monodontus*.

Chitwood and Chitwood (5) classified nematodes into two groups on the basis of the number of intestinal cells. Those which have less than 128 cells, the oligocytous nematodes, and those with over 128 cells, the polycytous nematodes. The Strongylina as a group appear to be oligocytous or low polycytous and the cells tend to be polynucleate. *D. stenocephala* is no exception to this rule and in the adult there were 32 intestinal cells, which, on cross section, were seen to be polynuclear. In the immature fifth state, however, the cells possess single large oval nuclei. These nuclei must therefore give rise to a number of smaller nuclei when the worms reach maturity. Chitwood and Chitwood (5) could give no definite reason for the polynuclear condition of the intestinal cells of the nematodes of this group. Apparently the increase in size of the individual cell increases the nuclear requirements of the cell so that a uninucleate cell is at a disadvantage. Many nuclei provide more nuclear surface and a closer association of cytoplasm with nucleoplasm than would be provided by a single nucleus.

The limited cell division that occurs in the gut cells is of interest. This limited cell division with remarkable constancy in cell numbers appears in other members of this group. Wetzel (27) and Lucker (17) have used this feature as an aid in the differentiation of the infective larvae of different species of horse strongyles. Dikmans and Andrews (7) have also made use of this in the differentiation of the infective larvae of sheep strongyles.

Observations on the development of the genital rudiment are very similar to those of Pai (19) on *Turbatrix aceti*, Alicata (1) on *Hyostrongylus rubidus*, and Schad (2) on *Kalicephalus parvus* and *K. rectiphilus*. Alicata (1) claimed that it was possible to determine the sex of larvae from the first stage onward by noting the position of the genital coelomocyte relative to the rudiment. Schad (21) was unable to show this in *Kalicephalus* and in the present work this was not established, mainly because of difficulty in ascertaining the location of the coelomocyte with certainty in all specimens examined.

Observations that the spicules develop from a pair of cell masses originating dorsal to the cloaca appears to coincide with those of Seurat (22) and Chitwood and Chitwood (5). Schad (21), on the other hand, found the spicular rudiment in parasitic third-stage larvae of *Kalicephalus* to be a single ovate mass lying dorsal to the rectum. He suggested that the primordium starts as a single mass and divides so that it is double in the fourth stage.

Coelomocytes have been described from widely divergent groups of nematodes and appear to be universal to this group of organisms. Chitwood and Chitwood (5) state that since the coelomocytes of all nematodes are homologous they must have similar if not identical functions and any differences

that have been noted may easily be of degree rather than character. They appear to be absorptive or phagocytic cells comparable to the fixed histiocytes of vertebrates. As the body fluids flow past them it is assumed that they have a purifying action.

Fulleborn's (10) contention that oral infection resulted in development without migration was substantiated. The oral route of infection appears to be the one of choice for the infective larvae, as attempts at cutaneous infection gave very poor results. It is possible that optimum conditions for skin penetration were not provided for the larvae. Spindler (24) has shown that the infective larvae of *Stephanurus dentatus* were able to penetrate the intact skin only when something was present on the skin against which the larvae could brace themselves. Perhaps this is the case with the larvae of *D. stenocephala*. Goodey (13) has shown that these larvae can penetrate the skin in an ensheathed condition if the drop of water containing them is allowed to dry on the skin. Law (14) obtained infection by placing infective larvae on the skin without covering them but gave no indication of the degree of infection obtained by this method. It is felt that the oral route of infection is the normal one. Oral infection results in heavy infection without the dangers inherent in somatic larvae migration, except for a transient migration into the lumen of the gastric and intestinal glands referred to as a "phylogenetic reminiscence" by Fulleborn (10). The histological response of the host to invasion by the larvae of this parasite has been described in a previous paper (11). It was observed that the cellular reaction provoked was of a very mild type in the gastrointestinal mucosa while in the lungs there was an acute inflammatory response. This indicates a pronounced resistance of the host tissues to invasion by the skin route. Foster and Cross (9) showed that the larvae of *A. caninum* were able to develop directly in the gut without somatic migration. In their studies development by the oral route of infection was 39.8% while by the cutaneous route it was only 7.5%. Likewise, Sarles (20) found that only 9% developed to adults when given cutaneously as opposed to 50% when given orally. Northern dogs, wolves, and foxes are heavily furred with a dense undercoat even between the toes; it therefore seems that the cutaneous route would be far more hazardous than the oral one. Furthermore, the marked habit or coprophagy shown by dogs would also facilitate infection by the oral route.

With increase in maturity of the larvae there was a gradual shift in the peaks of the larval distribution curves posteriorly along the small intestine at increasing distance from the pylorus. At maturity (15 days and 50 days) the greater number was found in the third quarter of the intestine. It has been the experience of Whitten (28) and this writer on previous occasions that the greater number of adults of this species was always found in the latter half of the small intestine. *D. stenocephala* adults therefore appear to favor this region of the small intestine as offering optimum living conditions.

Tetley (25) has demonstrated that in sheep the nematodes which infect the small intestine show a fairly constant preference for location and a normal distribution about this location, depending on the species. He concluded that, since neither active nor passive migration on the part of adult worms appeared to take place, the selection of an area of infestation must be the function of the incoming larvae. This general area of location of the adults must be deter-

mined by the rate of reaction of the larvae to continuously acting extrinsic stimuli. These stimuli are probably not contained in the walls of the intestine for the larvae would have to be in constant contact with the mucosa in order for the stimuli to act. The stimulus or stimuli must therefore be present among the contents of the stomach and small intestine and exert their effects from the time the larvae enter the stomach. This suggests as possible extrinsic stimuli the digestive enzymes or pH. De Beer *et al.* (6) found a pH gradient of 6.30 to 7.28 along the jejunum up to 7.61 for ileal juice in dogs and Shiffrin and Nasset (23) reported 6.9 to 8.6 to be pH range of jejunal and ileal secretion in dogs. This suggests that the pH of the gut contents is the likely stimulus. The gradual migration along the intestine coincident with maturity of the larvae would then be due to these larvae-seeking areas of higher pH migrating along the intestine away from the pylorus. That there is an inherent difference in response to the stimulus or stimuli between individual worms is evident because in some dogs examined a few apparently normal adult worms were found as far up as the latter portion of the duodenum.

In conclusion it appears that there is remarkable similarity in general structure not only between related species but among all the many species of nematodes. This is well shown in the work of Chitwood and Chitwood (5). However, sufficient differences exist between groups and sufficient similarities exist among the members of individual groups as to suggest that careful studies of developmental anatomy should be of value in further elucidating and confirming taxonomic relationships.

Summary

Studies were made on the development of the free-living and parasitic stages of *D. stenocephala*. The life cycle was typical of the ancylostomes. The ova were laid in the 8- to 16-cell stage and hatching was followed by development through three free-living larval stages. The third-stage larva (infective larva) was ensheathed and did not feed. Oral infection was more successful than percutaneous infection. After oral infection, somatic larval migration was unnecessary; the third-stage larvae penetrated the gastric and duodenal mucosal glands where they remained for up to 48 hours. Between 2 and 5 days the larvae attained the fourth stage and after about 7 to 8 days the fourth ecdysis occurred. After 15 days, mature ovigerous adults were present in the intestine.

Larvae with increase in maturity showed a definite migration pattern down the small intestine. The adults appeared to favor a location in the latter third or fourth of the small intestine.

Development of the organ systems appeared to parallel closely that described for other strongyles.

A brief description of the anatomy of the adult has been given.

Acknowledgments

The author is indebted to Professor T. W. M. Cameron, Director, Institute of Parasitology, McGill University, under whose direction this study was undertaken. Thanks are due to Mrs. Jessie Shah, who prepared the sections of adult worms for histological study.

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A COMPARISON OF INJURY AND SURVIVAL OF LARVAE OF CEPHUS CINCTUS NORT. AFTER INTRACELLULAR AND EXTRACELLULAR FREEZING[1]

R. W. SALT

Abstract

Extracellular freezing of larvae of the wheat stem sawfly, *Cephus cinctus* Nort., was produced at −2.5° C by a new method. Slow further cooling to −10, −15, or −20° C added to extracellular ice with no intracellular freezing. Other larvae that were supercooled to and frozen at −10, −15, or −20° C froze intracellularly. Comparisons of the effects of these two types of freezing were therefore possible at equivalent temperatures. Level of activity after freezing was used as the criterion of injury.

Intracellular freezing was more injurious than extracellular freezing at −15 and −20° C, but not at −10° C. Injuries, as well as differences in injury due to type of freezing, decreased gradually to insignificance above −10° C. Although larvae frozen extracellularly held an initial advantage over those frozen intracellularly, survivors of the latter group retained their vitality better, probably because they lost weight more slowly.

Differences in injury and in activity level after freezing at −15 and −20° C were insufficient to justify the use of freezing site (intracellular or extracellular) as a principal basis for explaining freezing injury. The same conclusion applies to ice crystal size and configuration, which differed vastly in the two types of freezing.

These conclusions depend on whether freezing was actually intracellular or extracellular as represented. Strong evidence is presented that freezing was in fact as specified.

Introduction

Intracellular freezing is generally regarded as fatal to both animal and plant cells (1, 2, 3, 5, 6). There is considerable experimental evidence supporting this opinion and there seems little doubt that conditions giving rise to ice formation within a living cell are usually harmful to the organism unless protective substances such as glycerol are present. Extracellular freezing may also be lethal, but in some circumstances, either natural or artificial, it is essentially harmless. Extracellular freezing initiated near the freezing point, followed by slow cooling to produce slow extraction of cellular water, is often innocuous if the temperature is not further reduced too much nor maintained too long. Thus extracellular freezing produces an injury response graded from harmless to fatal. On the other hand, if intracellular freezing is as harmful as generally thought, it must produce either a total response (sudden death) or one restricted to a "severely injured" range.

The writer has long been of the opinion that the freezing of whole insects is of the intracellular type whenever it follows supercooling of a few degrees or more, which is almost always the case. It has been a common observation, moreover, that freezing of insects following moderate supercooling produces

[1]Manuscript received March 8, 1961.
Contribution from the Entomology Section, Research Station, Research Branch, Canada Department of Agriculture, Lethbridge, Alberta.

injuries that are graded in direct proportion to exposure, just as with extracellular freezing. This can be demonstrated and is not open to question, but proof that freezing is, in fact, intracellular is more difficult. This paper presents a comparison of the extent of injuries suffered by whole insects that were frozen in two different ways, and evidence that intracellular freezing was produced by one method and extracellular freezing by the other. All mention of cells in this paper is confined to those not protected by glycerol or other such substances.

Materials and Methods

Larvae of the wheat stem sawfly, *Cephus cinctus* Nort., that had been stored at 0° C for several months were removed from wheat stubble just before treatment.

Temperatures, given in Centigrade degrees throughout this paper, were controlled by moving a horizontal metal plate up or down in the temperature gradient of a refrigerated chamber and were monitored by a thermocouple and recording potentiometer. Each sample of 50 larvae was placed on the plate and brought to a temperature of −2.5 to −3.0°. Twenty-five larvae were then frozen by means of a new method, described below, and after 2 or 3 minutes were checked for hardness to ensure that all were frozen. The temperature was then lowered slowly to −10, −15, or −20° over a period of 1 hour, half of this time being spent between −3 and −6°. At −10, −15, or −20° freezing was induced in the remaining larvae and the temperature was held constant for 1 hour. The entire sample of 50 larvae was then thawed at room temperature and kept there at 90% R.H. The activity of each individual was rated daily for 5 days. Six replications were made at each temperature.

Activity ratings were chosen with regard for the fact that every larva had been frozen and had probably suffered some slight injury at least. Hence the top rating did not necessarily indicate total lack of injury. Larvae that squirmed of their own accord rated 4; those that squirmed only when prodded rated 3; those exhibiting less movement, down to the slightest muscle twitch, rated 2; any visible heart beat, in the absence of other muscular movement, rated 1; no movement or heart beat rated 0. The ratings for each set of larvae were averaged daily over the 5-day observation period, and a comparison was also made on the basis of the best rating attained by each larva.

The method used to induce freezing is new and made the experiment possible. The usual method of freezing water on the cuticle often fails to inoculate at temperatures above −10° and is quite ineffective above −5°. Instead, a high voltage current, which induced nucleation and freezing even at temperatures close to the freezing point, was used (7). A spark coil from a model T Ford automobile was energized by the smallest potential (1.5 to 2 volts) that would produce a hum in the vibrator. The insects lay on one electrode, the cold plate, and were touched one at a time by the other electrode, a probe. No spark was observed under these minimal conditions. Freezing at temperatures near the freezing point was more certain with this procedure than with the original method described in reference 7, where a greater input voltage and an electrode separation of $\frac{1}{2}$ inch created an electrostatic field that induced freezing.

Evidence on Site of Freezing

The freezing that was started at −2.5 to −3.0° and followed by slow cooling is considered to be entirely extracellular, whereas that induced after supercooling to −10, −15, and −20° is considered to be intracellular. The evidence for these conclusions follows.

(a) Tissue Frozen in Ringer's Medium

Pieces of salivary gland and fat body with small amounts of Ringer's solution were cooled on a freezing stage of a stereoscopic microscope. These tissues have large cells that can be individually observed. Freezing of the medium can be readily obtained at its freezing point by inoculation with an ice crystal; without inoculation, supercooling of a few degrees up to about 10 degrees is obtained before freezing. With the former procedure, ice formation throughout the medium is clearly observed, but there is never any indication of ice inside the cells. Contraction and distortion of the cells is seen, but the over-all dimensions of the tissue may not be reduced (Figs. 1*a*, 1*b*). Most of this effect, which is caused by the loss of water from the cells, is seen in the first stages of cooling, as expected, and thereafter little further change is seen. Transparency is not noticeably reduced. When cooling is slow enough to maintain near-equilibrium ice formation, the freezing point of the cell contents decreases with, and is very nearly the same as, the falling temperature. Hence there is almost no possibility of the cells freezing when cooling is this slow.

When cooling is more rapid, one observes a sudden darkening of some or all of the cells (Fig. 1*c*). This is a common observation and has been described and illustrated by Asahina (1), who calls it "flashing". It indicates freezing inside the cell. It also indicates something about the speed with which water can leave the cell to freeze as extracellular ice. Under cooling conditions that we have called slow, the time allowed for water to leave the cell was sufficient that the falling freezing point of the intracellular liquids kept pace with the falling ambient temperature. Under faster cooling conditions, water loss from the cells presumably does not keep pace with the falling temperature, for in order to freeze internally the cell contents must be more dilute than the extracellular liquids, have a higher freezing point, and thus be supercooled. Hence a critical rate of cooling lies between the two rates we have described, separating the incidence of extracellular and intracellular freezing.

An interesting sidelight on the foregoing is concerned with the role of pore diameter in determining the temperature at which ice will penetrate the cell from without (4). Slow cooling conditions produce only extracellular freezing, without penetration by ice into the cells; this is consistently observed to be the case down to at least −25°. It indicates a small pore size allowing penetration by ice only at temperatures somewhere below −25°. We have also seen, however, that a more rapid temperature drop, even at fairly high temperatures, gives rise to intracellular freezing. This cannot be attributed to pore penetration, which did not occur at these temperatures when cooling was slow, unless in the latter circumstances the loss of water progressively shrank the pores below their critical diameter for ice penetration, an unlikely explanation.

(b) Whole Frozen Larvae

We can control conditions on a freezing stage to produce at will either intra-

cellular or extracellular freezing in a preparation consisting of tissue and liquid medium. Can this also be done with whole organisms? The newly discovered technique of nucleation by electrical means allowed whole larvae of *C. cinctus* to be frozen within 1 or 2 degrees of their freezing point, ice formation being very probably extracellular. Slow further cooling ensured continued extracellular freezing. Other larvae, supercooled 10 degrees or more and then frozen, were assumed on the basis of the tissue observations to be frozen intracellularly. The validity of these assumptions regarding site of freezing is supported by considerable visual evidence. Whole larvae frozen at −2.5 and cooled slowly to −10° look much different from larvae supercooled to and frozen at −10°. The former have an amber translucency when viewed with obliquely transmitted light; the latter have an opaque, milky-white appearance. The difference, quite striking to the eye, is depicted reasonably well by the camera in terms of density (Fig. 1*d*).

(c) Dissection After Freezing

When whole, frozen larvae were dissected, the differences in freezing type were particularly clear (Fig. 1*e*). The dissections were made in mineral oil at controlled subfreezing temperatures under a dissecting microscope, using knife-edged needles and small fragments of razor blades as implements. Light was transmitted obliquely through the specimens from below.

Larvae frozen at −2.5 and cooled slowly to −10° or colder are essentially transparent, even in sections as thick as one-fourth of the larval diameter. A dominant feature is the abundance of large crystals of clear ice, variable in shape but essentially prismatic, and forming single rather than ramified, dendritic, or other such compound units. Such ice appears to occupy the entire body cavity except for the fat body, salivary glands, gonads, neural ganglia, and the slim, tubular gut. These tissues are not crystal clear but are nevertheless transparent, so that one can readily see into them, especially into large cells such as salivary gland cells and fat cells. Muscles are completely transparent, so that along the dorsum, for example, the cuticle, muscles, and heart appear to merge into a clear undifferentiated mass of ice crystals whose long axes parallel that of the larva. The other tissues are best seen in larvae that have been injected with a dye such as fast green. When this is done the ice is clear, as are the muscles, cuticle, hypodermis, and tracheae; the ganglia and gut stain green, the fat body moderately so, and the salivary gland very lightly. At temperatures down to about −10° it is quite certain that the cellular tissues are unfrozen, for if the ice is teased away from them, they are completely fluid. At lower temperatures this technique becomes more difficult, for with more ice and less unfrozen liquid separating it from the tissues and from other ice crystals, a more cohesive and harder mass is produced. Nevertheless, one can with care dissect out bits of tissue at −15° and observe by touch as well as by their transparency that they are unfrozen. There is no reason to doubt that their continued transparency when cooled to much lower temperatures indicates that they remain unfrozen.

A technique that appears obvious and appropriate at this point will be described because it illustrates one of the pitfalls that are common in this type of research. Since dissection becomes difficult below −10° it seems unobjection-

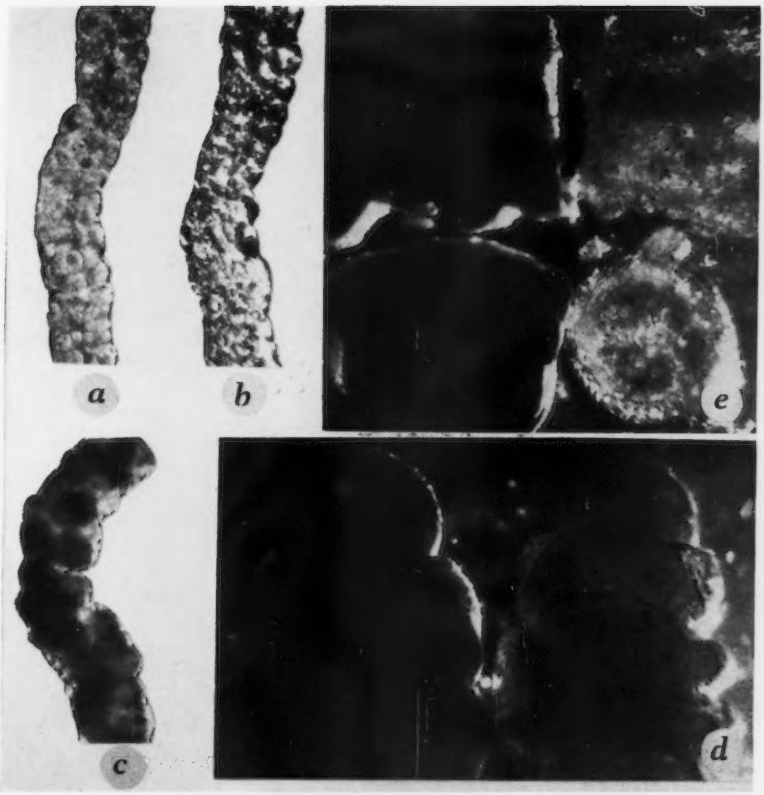

FIG. 1. Tissues, sections, and whole larvae of *Cephus cinctus*. (*a*) Salivary gland in Ringer's solution, before freezing. (*b*) Same tissue after preparation frozen extracellularly; medium inoculated at −1°, cooled slowly. Tissue dehydrated but unfrozen. Photographed at −25°. (*c*) Salivary gland frozen intracellularly by supercooling to and freezing at −5°. Photographed at −8°. (*d*) Larva on left frozen intracellularly by supercooling to and freezing at −10°; larva on right frozen extracellularly at −2.5°, cooled slowly to −10°. Photographed at −10°. (*e*) Vertical (upper) and cross (lower) sections of dyed larvae frozen as in (*d*). Freezing intracellular on left, extracellular on right. Photographed at −10°.

able to dissect out a tissue at a fairly high temperature and then cool it slowly. However, in the undissected organism the unfrozen tissue is separated from the adjacent extracellular ice by a film of unfrozen liquid, usually haemolymph. When, in a dissection under oil, the ice is pulled away from the tissue, both ice and tissue are left with a film of unfrozen haemolymph separating them from the oil. If the temperature is now lowered, this film will freeze to an extent dictated by its concentration and temperature. Hence a skin of ice will form on the outside of such a dissected tissue, and its rigidity may delude one into concluding that the tissue itself is frozen. (If the tissue were completely free of ice, the film would probably supercool, but under usual conditions of dissection some ice is certain to adhere and inoculate the film.)

When dissections are made of larvae that have been supercooled 10 to 20 degrees before freezing, the fine-grained structure, increased hardness, and large reduction in transparency are especially noticeable. The frozen tissues break into minute crystals when prodded with a fine point, and although it is not certain whether these are discrete units or fragments of a compound dendritic crystal structure, their small size contrasts greatly with the large prisms produced by extracellular freezing. The interference with light transmission caused by the innumerable crystal faces covered with films of concentrated, unfrozen haemolymph produces a translucency, which in bulky cellular tissues amounts almost to opacity. Of course, these terms are relative, being dependent on the thickness of the section or fragment observed. If a section is thin enough to allow a fair amount of light through the frozen haemolymph, the muscles will appear somewhat denser, and the other tissues considerably more dense, but both distinguishable from their surroundings in gross aspect. If most of the ice can be removed from around a piece of tissue, the latter is found to be hard and frozen at temperatures of $-10°$ and lower. Fragments of salivary gland demonstrate this well, as the large spheroidal cells chip out of the frozen tissue as discrete, hard units. Crushing these produces minute crystals or fragments which scintillate as they disperse in the oil. Ganglia show a similar effect, but are multicellular and the individual cells cannot be seen. In addition to this evidence, individual salivary gland cells have the granular, semi-opaque appearance that is characteristic of internal freezing as already described for tissues supercooled and frozen in a liquid medium. Fat cells, also large, show the same characteristic opacity somewhat modified by the presence of transparent oil droplets within them.

The above observations are readily made at $-10°$ and with some difficulty at $-15°$. If the temperature is raised to $-5°$, the rigidity or hardness of the frozen cells becomes diminished and difficult to appraise because the melting at each crystal face allows slippage under pressure, or in other words, slushiness. The opacity of the cells, however, is almost as great as at lower temperatures. Freezing that follows supercooling of only a few degrees produces this same opacity, and intracellular freezing can be shown to have taken place through the evidence of intracellular hardening detectable when the specimen is further cooled.

In summary, intracellular and extracellular freezing can be produced at will in tissue-and-medium preparations or in whole larvae, and the authenticity

of either type of freezing can be demonstrated by tests based on light transmission, cell hardness, and ice crystal size. Consequently, the experiments reported herein are considered to represent the two types of freezing precisely as specified.

Results

Comparison of relative survival and activity after the two types of freezing, which was the primary purpose of the experiment, is summarized in Table I. The ratings of the six replicates are averaged, whereas the *t* values are derived from paired replicates. Intracellular freezing was definitely more injurious than extracellular freezing at −15 and −20°, but not at −10°, a conclusion arrived at with either system of rating. The quantitative difference in injury apparently fades out above −10° and here also the possibility of freezing being intracellular declines. At −10° the injuries are slight, regardless of freezing type, and it is apparent that they would decrease further with rising temperature. Moreover, since the injuries are slight, differences in them are small and must approach insignificance in this temperature range. This they are seen to do, and the fact that observed differences are in the same direction at −10° as at −15 and −20° suggests that they are indeed present even though not statistically significant.

An extension of the experiment to −5° would have been of value, but the uncertainty of obtaining intracellular freezing with confidence at this temperature precluded this. Nevertheless, it appears that near the upper limit of intracellular freezing the quantitative aspects of the two types of freezing injury merge and approach zero or minimum values.

The day-to-day trends of activity following treatment are shown in Fig. 2. The larvae deteriorated, on the average, after treatment at −15 and −20°, and possibly after treatment at −10°. One reason for deterioration became apparent when, after 5 days of observation, a few larvae were found to be rather dry. To investigate this is more detail, two sets of 25 larvae were frozen at or to −10° C, one set intracellularly and the other extracellularly, and kept at room temperature and humidity (about 23° and 20% R. H.). Figure 3 shows that larvae frozen extracellularly lost weight more readily, especially

TABLE I

Comparison of average and best activity ratings of *C. cinctus* larvae during a 5-day period following intracellular or extracellular freezing at −10, −15, and −20° C. Average of six replicates. *t* values derived from paired replicates

Temp. (°C)	Intracellular freezing	Extracellular freezing	*t*
	5-Day average rating		
−10	81	83	0.42
−15	30	52	7.46*
−20	7	20	5.20*
	Best rating		
−10	89	94	1.15
−15	45	70	7.70*
−20	18	34	4.92*

*Significant at the 1% level.

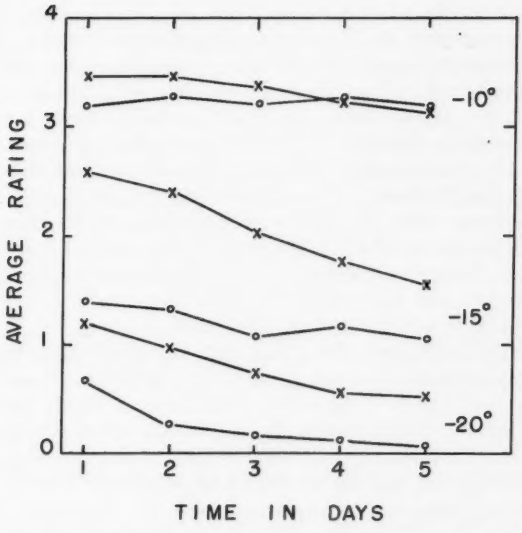

FIG. 2. Average activity ratings of *C. cinctus* larvae during first 5 days after having been frozen intracellularly (○) or extracellularly (×). Kept at room temperature and 90% R. H.

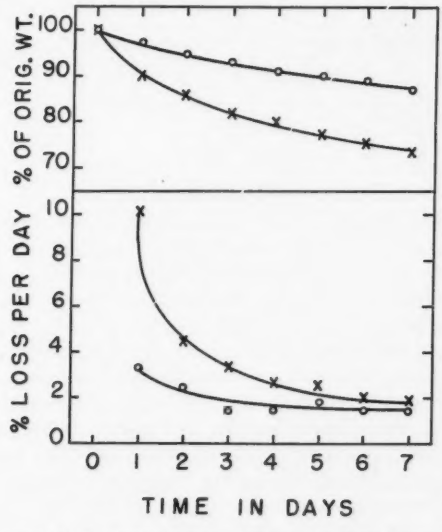

FIG. 3. Weight losses of *C. cinctus* larvae after having been frozen intracellularly (○) or extracellularly (×). Kept at room temperature and humidity (about 23° and 20% R. H.). Upper curves show decreasing weights, lower curves daily losses.

in the first few days, than larvae frozen intracellularly. In the main experiment larvae were kept at 90% R. H. and lost weight much more slowly; they perhaps soon recovered their prefreezing level of resistance to water loss, for Fig. 3 shows also that the rate of loss subsided to a low level after a few days.

Table II lists the percentages of larvae that maintained an "active" rating for 5, 28, and 70 days after freezing. Of these, the larvae frozen intracellularly maintained their activity longer than those frozen extracellularly. After treatment at −10° equal numbers were active on the 5th day, but whereas all of these that were frozen intracellularly were still active on the 28th day, the others were reduced in numbers by 43%. By the 70th day the relative advantage was almost 3 to 1 in favor of intracellular freezing. After treatment at −15° the same pattern exists, but with a much different beginning. At this temperature the extracellularly frozen larvae had an initial advantage of more than 3 to 1 on the 5th day, deteriorated thereafter, but retained a net advantage on the 28th day. It seems, therefore, that larvae frozen intracellularly retain their vitality better than larvae frozen extracellularly, probably because they lose weight more slowly. In summary, larvae frozen intracellularly are injured as much at −10° C, and more at lower temperatures, as compared to extracellularly frozen larvae, but the survivors subsequently fare better.

TABLE II

Percentages of larvae rating "active" 5, 28, and 70 days after freezing

	5 days*		28 days†		70 days†	
Temp. (°C)	I‡	E§	I	E	I	E
−10	72	73	72	39	36	13
−15	7	23	5	9	0	0
−20	0	1	0	0	0	0

*Six replications of 25 larvae each.
†Three replications of 25 larvae each.
‡Intracellular freezing.
§Extracellular freezing.

Discussion

The terms "intracellular" and "extracellular" are very useful and convenient, clearly descriptive, and relatively unambiguous. When freezing is termed extracellular it is implied that this is the only type present. It is possible to add intracellular freezing by cooling more rapidly than water can leave the cells to maintain osmotic equilibrium, thus causing the cell contents to become more dilute than the extracellular medium and hence to be supercooled and then frozen; alternatively, the temperature may go low enough to permit ice crystals to grow through the cell pores (4), though my observations do not support this idea. When freezing is termed intracellular, on the other hand, it is assumed that extracellular liquids have also frozen at about the same time, and there is no implication nor reason to believe that they enter the cell to be added to intracellular ice. Generally, the terms are used to distinguish whether or not the cell contents are frozen.

The experimental results show an initial advantage to extracellular freezing (relative to intracellular freezing) at temperatures below −10°, but one which

is short-lived, since larvae frozen intracellularly retain their vitality better when they survive at all. At temperatures of $-10°$ and higher, where there is some possibility of recovering from freezing injury, there is no significant relative advantage to either type of freezing at first, but subsequently intracellular freezing proves the less injurious.

A great deal of importance is sometimes attached to the site of freezing and also the size and configuration of the ice that is formed. In the present experiments, greatly contrasting freezing sites and crystallization patterns were produced, yet the differences in injury were small. It is quite clear that neither of these two physical aspects of freezing is of such paramount importance that it would legitimately form by itself the basis of a theory of freezing injury.

Acknowledgments

Criticisms of the manuscript by Doctors N. S. Church, H. J. Perkins, and T. G. Atkinson of the Research Station, Lethbridge, Alberta, are gratefully acknowledged.

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MARINE NEMATODES FROM THE COAST LINE OF THE GULF OF MEXICO, II[1]

B. E. HOPPER

Abstract

Marine nemas from the Alabama coast of the Gulf of Mexico are recorded. *Metachromadora* (*Neonyx*) *obesa* Chitwood, 1936 is redescribed. *Hypodontolaimus pandispiculatus*, *Rhynchonema hirsuta*, and *Theristus* (*Theristus*) *rhynchonemoides* are described as new. *Rhynchonema cinctum* of Wieser, 1956, nec Cobb, 1920, is renamed *R. wieseri* n. nom. *Rhynchonema cinctum* of Gerlach, 1955 and Wieser, 1959, nec Cobb, 1920, are considered synonyms of *R. hirsuta* n. sp. A key to the species of the genus *Rhynchonema* is provided.

The material in this article represents the second in a series of papers on the marine nemic fauna from the Alabama coast line of the Gulf of Mexico. Four species are added to the Alabama fauna, of which three are regarded as being new to science. In addition, the genus *Rhynchonema* Cobb, 1920 is reviewed.

General Remarks

All samples came from the vicinity of Gulf Shores, Alabama. All observations were made on preserved specimens mounted in glycerine on Cobb slides. The spicular length recorded herein is the absolute spicular length and not the cord of the spicular arc. The types of all new species described are in the possession of the author.

List of Species and Name Changes

Family Desmodoridae
  Subfamily Richtersiinae
    Metachromadora (*Neonyx*) *obesa* Chitwood, 1936
Family Chromadoridae
  Hypodontolaimus pandispiculatus n. sp.
Family Monhysteridae
  Rhynchonema hirsuta n. sp.
    Synonym: *R. cinctum* Cobb of Gerlach, 1955
    " *R. cinctum* Cobb of Wieser, 1959
    nec: *R. cinctum* Cobb, 1920
    " *R. cinctum* Cobb of Wieser, 1956
  Rhynchonema wieseri n. nom.
    Synonym: *R. cinctum* Cobb of Wieser, 1956
    nec: *R. cinctum* Cobb, 1920
    " *R. cinctum* Cobb of Gerlach, 1955
    " *R. cinctum* Cobb of Wieser, 1959
  Theristus (*Theristus*) *rhynchonemoides* n. sp.

[1]Manuscript received February 21, 1961.
Contribution from the Nematology Section, Entomology Research Institute, Research Branch, Canada Department of Agriculture, Ottawa.

Description of Species

Family DESMODORIDAE (Filipjev, 1922) Micoletzky, 1924
Subfamily RICHTERSIINAE Kreis, 1927 Cobb, 1933
Metachromadora Filipjev, 1918
Metachromadora (*Neonyx*) *obesa* Chitwood, 1936 (Fig. 13)

Female (*n*, 1).—Length = 1.26 mm; $a = 13.9$; $b = 5.7$; $c = 13.2$; $V = 54\%$.

Body stout, 90 μ wide. Cuticle finely striated, with prominent lateral alae. Body bearing six rows of somatic setae, a dorsal row 10 μ long, a ventral row 10 to 13 μ long, and four sublateral rows 15 to 20 μ long.

Head with four cephalic setae, 10 μ long, their length one third the head diameter. Amphid 7.5 μ wide. Stoma without denticles, with large, spear-like onchium. Esophagus 220 μ long with tripartite terminal bulb, 90 μ long by 50 μ wide, with heavily sclerotized lining.

Setae in vulvar region subventrally paired 13 μ long. Anal body diameter 40 μ. Tail 95 μ long.

Remarks.—*M.* (*N.*) *obesa* from the Gulf of Mexico, except for being the longest specimen recorded to date, agrees well with the type description and that of Timm (16).

Specimen.—One female on slide M26.

Occurrence.—Collected by author February, 1957. Specimens washed from mud and silt collected in tidal pond. Habitat sheltered. Salinity 18‰.

Family CHROMADORIDAE Filipjev, 1917
Subfamily CHROMADORINAE [Filipjev, 1917] Micoletzky, 1922
Hypodontolaimus de Man, 1886
Hypodontolaimus pandispiculatus n. sp. (Figs. 1–4)

Female (*n*, 5).—Length = 1.04 (0.97–1.07) mm; $a = 18.3$ (17.6–19.3); $b = 7.0$ (6.5–7.7); $c = 8.0$ (7.3–8.6); $V = 46.5$ (44.7–49) %.

Male (*n*, 6).—Length = 1.01 (0.97–1.14) mm; $a = 20.1$ (17.7–22.8); $b = 6.8$ (6.6–7.2); $c = 8.8$ (7.4–12.2).

Body spindle-shaped, 50 to 57 μ wide. Cuticular ornamentation homogeneous, differentiated by lateral alae. Lateral alae bordered by two rows of heavier punctations. Cervical and somatic setae, 7 to 9 μ long, arranged in two rows, one on either side of lateral alae.

Head 15 μ wide, armed with four cephalic setae, 6 to 7 μ long. Stoma of typical structure (cf. Wieser (17)). Pharyngeal musculature 9 μ wide and 15 μ long. Esophagus terminated by bipartite bulb, 46 μ long by 31 μ wide. Excretory pore anteriorly placed, opposite dorsal tooth. Excretory duct leading into large ampulla (Fig. 7). Renette cell posterior to esophagus. Neck base diameter, 42 to 45 μ.

Anal body diameter 32 to 35 μ. Tail of male curled ventrally when relaxed by heat. Spicules 40 μ long, strongly bent in middle. Gubernaculum about one half spicular length with small dorsal apophysis (Fig. 4). No supplements.

Differential diagnosis.—*Hypodontolaimus pandispiculatus* n. sp. is most closely related to *H. ponticus* Filipjev, 1922 but differs from it in having longer body measurements, shorter cephalic setae, and sharply bent spicules. Follow-

ing the key given by Wieser (17), *H. pandispiculatus* n. sp. keys out to *H. geophila* (de Man, 1876). It is distinguished from *H. geophila* by having a two-sectioned esophageal bulb and a small apophysis on the gubernaculum, and by the sharply bent nature of the spicules. The spicules of both *H. ponticus* and *H. geophila* are uniformly arcuate (cf. Gerlach (9), Plate 31, Fig. 3*d*, and Plate 38, Fig. 14*d*).

Holotype.—Male on slide M5.

Allotype.—Female on slide M5.

Paratypes.—Five females, six males on slide M5; one male, three juveniles on slide M4.

Occurrence.—Collected February, 1957, and September, 1958, by author. Specimens washed from sand collected in tidal pond. Habitat sheltered. Salinity 15.7‰.

Family MONHYSTERIDAE de Man, 1876

Subfamily MONHYSTERINAE (de Man, 1876) [Filipjev, 1918] Micoletsky, 1922

Rhynchonema Cobb, 1920

Up to the present time the information on the genus *Rhynchonema* Cobb, 1920 is scant, there being only four species described for the genus with two redescriptions of the type, *R. cinctum* Cobb, 1920, and one of *R. lyngei* (Allgen, 1940) Gerlach, 1953.

R. lyngei was first described by Allgen as being a species of the genus *Leptolaimus* de Man, 1876. As Gerlach (11) points out, the description was primarily a comparison with the other species of *Leptolaimus* and thus rather insufficiently characterized for comparison with *Rhynchonema*. *Leptolaimus lyngei* Allgen, 1940 was described from a single female specimen measuring 0.835 mm long. Gerlach records the length as 0.596 mm. In addition, Gerlach illustrates numerous long cervical setae which Allgen fails to mention. The correct relationship between Allgen's and Gerlach's specimens remains uncertain until such time that further information is available regarding the variability of body length and pilosity. It has been this author's observation that body length is quite variable within the species, but that body structures are more constant.

A study of specimens collected from the Gulf of Mexico and a re-evaluation of the characters reported in the redescriptions of *R. cinctum* has led to the conclusion that each description represents a different species. Gerlach (12), in recording *R. cinctum* from San Salvador, states that the agreement between the Central American and the Peruvian animals is not complete and lists as one discrepancy the differences in stoma length. *R. cinctum* Cobb of Gerlach, 1955 is herein regarded as a synonym of *R. hirsuta* n. sp. Wieser (19) also records an *R. cinctum* Cobb from Puget Sound beaches (without description or figures), stating that the single female specimen "in every respect conforms with the description of this species by Gerlach (12)". The writer therefore considers that Wieser, also, probably had material of *R. hirsuta* n. sp. *R. cinctum* Cobb of Wieser 1956 differs from both *R. cinctum* Cibb, 1920 and *R. hirsuta* n. sp. and accordingly is renamed *R. wieseri* n. nom.

Rhynchonema hirsuta n. sp. (Figs. 5–8)
Synonym: *R. cinctum* of Gerlach, 1955
" *R. cinctum* of Wieser, 1959
nec: *R. cinctum* Cobb, 1920
" *R. cinctum* Wieser, 1956

Female (*n*, 5).—Length = 0.533 mm (.525–.550); $a = 21.3$ (21–22); $b = 3.94$ (3.75–4.0); $c = 6.58$ (6.1–6.8); $V = 64.1$ (60.1–66.7) %.

Male (*n*, 1).—Length = 0.533 mm; $a = 21.3$; $b = 4.1$; $c = 6.7$.

Body (Figs. 7, 8) anterior to neck base, at level of amphid, greatly narrowed forming beak-like neck region. Body posterior to neck base of near uniform diameter (25 μ) to anal region. Anal body diameter 21 μ. Tail gradually narrowed, conoid with terminal spinneret. Cuticle strongly annulated, annules retrorse posterior to vulva, the reverse anteriorly, with numerous scattered somatic setae up to 17–21 μ long.

Head bearing six small papillae about the mouth opening and six 3–4 μ long, cephalic setae about one head diameter posterior to anterior extremity (Fig. 5). Stoma 2 μ wide by 53–55 μ long. Amphid in male 6 μ, in female 4 μ wide, located opposite base of stoma. Esophagus conoid, 6 μ wide at beginning to 16 μ wide posteriorly.

Ovary monodelphic, prodelphic, outstretched. Spicules 26 μ long, arcuate at proximal cephalation (Fig. 6). Gubernaculum small, flanking spicula on both sides, with caudally directed apophysis.

In front of the anus, on the ventral side, there appear to be five breaks in the hypodermis and musculature. The three anterior are clearly supplementary organs, while the remaining two perhaps represent rudiments of such organs. There are no external indications of these supplements, i.e., no papilloid structures as described by Cobb for *R. cinctum*. Their distances anterior to the cloacal opening are 10 μ, 20 μ, 35 μ, 50 μ, and 65 μ. In total they occupy a distance equal to 2.2 body diameters and the anterior four are separated from each other by three fifths of a body diameter.

Differential diagnosis.—*Rhynchonema*, most closely related to *R. lyngei* (Allgen, 1940) Gerlach, 1953. Differing in that (i) the location of the amphids is at the base of the stoma, while those of *R. lyngei* are posterior to the stoma, (ii) there are only six cephalic setae as against 10 for *R. lyngei*, and (iii) the spicules are shorter (26 μ: 32 μ) and are not as arcuate as in *R. lyngei*.

The only difference between *R. hirsuta* n. sp., and *R. cinctum* of Gerlach, 1955 is the length of the spicules, 26 μ and 21 μ respectively. As only a single male specimen of each is known, their exact relationship is dubious.

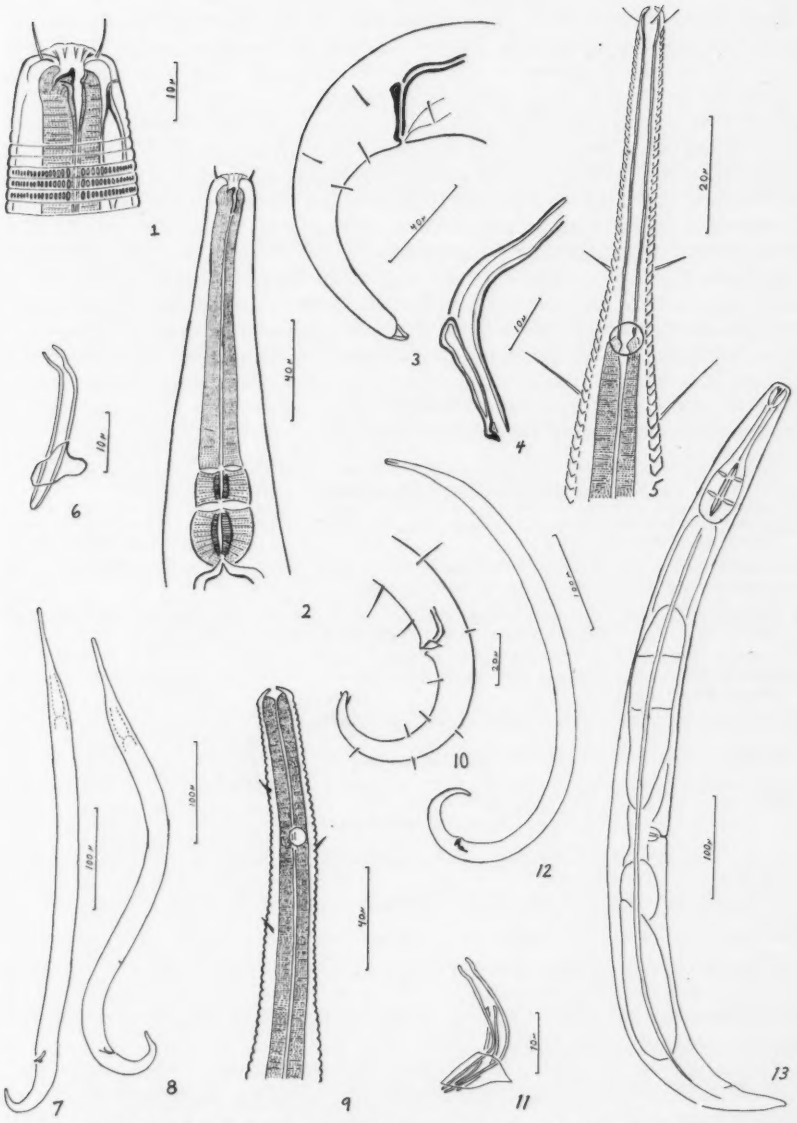
Holotype.—Male on slide M40.

Allotype.—Female on slide M40A.

Paratypes.—Three juveniles on slide M40B; eight females on slide M7.

Occurrence.—Collected May, 1958, by N. Sansing. Specimens washed from sand collected below low tide mark. Habitat exposed. Salinity 26.9‰.

Rhynchonema wieseri nov. nom.
Synonym: *R. cinctum* of Wieser, 1956
nec: *R. cinctum* Cobb, 1920
" *R. cinctum* Wieser, 1959
" *R. cinctum* Gerlach, 1955



FIGS. 1–4. *Hypodontolaimus pandispiculatus* n. sp. 1. Head of female. 2. Esophageal region of male. 3. Male tail. 4. Spicules and gubernaculum.

FIGS. 5–8. *Rhynchonema hirsuta* n. sp. 5. Head of female. 6. Spicules and gubernaculum. 7. Body form of male. 8. Body form of female.

FIGS. 9–12. *Theristus* (*Theristus*) *rhynchonemoides* n. sp. 9. Head of male. 10. Male tail. 11. Spicules and gubernaculum. 12. Body form of male.

FIG. 13. *Metachromadora* (*Neonyx*) *obesa* Chitwood. Body form of female.

Differential diagnosis.—*R. wieseri* nov. nom., is closely related to *R. cinctum* but differs in several respects. The major differences appear to be the length of the stoma and the shape of the anterior extremity. The stoma in Cobb's *R. cinctum* is 36–37 μ long, while in *R. wieseri* the stoma is 44 μ long. The stomatal region in Cobb's species is nearly cylindrical, the diameter at the base of the stoma being about 1.7 head diameters wide (measured at level of cephalic setae), while the diameter at the base of the stoma in *R. wieseri* is three times the width of the head. Furthermore, the annules are proportionately wider in *R. wieseri*, only 25 being present between the anterior extremity and the base of the stoma, while there are approximately 30–35 in *R. cinctum* (estimated from Cobb's drawing). The anterior edge of the amphid lies at the very base of the stoma in *R. wieseri*, while in *R. cinctum* it lies about its own diameter posterior to the base of the stoma. *R. wieseri* appears to bear many cervical setae just posterior to the amphid whereas Cobb illustrates only one in his Fig. 43. In addition, Cobb makes no mention of somatic setae being present, while Wieser's specimens have "somatic setae up to 13 μ long, arranged in apparently 8 longitudinal rows".

Key to the Species *Rhynchonema* Cobb, 1920

1. Stoma less than 45 μ long 2
   Stoma more than 50 μ long 4
2. Amphids opposite posterior end of stoma, stoma 27–33 μ long ..... *brevituba* Gerlach, 1952
   Amphids posterior to stoma, stoma 36–44 μ long 3
3. Stoma 36–37 μ long; diameter at base of stoma 1.8 head diameters wide ..... *cinctum* Cobb, 1920
   Stoma 44 μ long; diameter at base of stoma 3.5 head diameters wide ..... *wieseri* nov. nom. (Syn. *cinctum* of Wieser, 1956)
4. Stoma 65–67 μ long ..... *longituba* Gerlach, 1952
   Stoma 52–60 μ long 5
5. Amphids opposite posterior end of stoma; six cephalic setae ..... *hirsuta* n. sp. (Syn. *cinctum* of Gerlach, 1955)
   Amphids posterior to stoma; 10 cephalic setae ..... *lyngei* (Allgen, 1940) Gerlach, 1953 (Syn. *Leptolaimus lyngei* Allgen, 1940)

Theristus Bastian, 1865

Theristus (*Theristus*) *rhynchonemoides* n. sp. (Figs. 9–12)

Female (*n*, 1).—Length = 0.53 mm; a = 23.3; b = 4.8; c = 5.8; V = 63.3%.

Male (*n*, 4).—Length = 0.59 (0.54–0.62) mm; a = 29.6 (26.7–30.7); b = 5.1 (4.5–5.5); c = 6.7 (6.2–7.2).

Body with form of *Rhynchonema* (Fig. 12). Head diameter 6 μ, neck enlarging rapidly in middle attaining a width of 19 μ at its base. Body behind neck base of uniform diameter (19 to 21 μ) to anal region. Tail conoid, tapering uniformly, ventrally arcuate.

Cephalic setae not observed (missing or minute?). Amphids 2.8 μ wide, thin-walled, indistinct, seen in only one male, located 25 μ (four head diameters) behind front end.

Cervical setae on narrow portion of neck 3 μ, on expanded region, 15 μ long. Body with numerous somatic setae varying from 12 to 23 μ in length. Caudal setae 15 μ long. Tail with two minute terminal setae which are less than 2 μ long.

Spicules asymmetrical, the right one 16 μ long, with a gradual arc, the left 26 μ long and strongly flexed (Fig. 11). Gubernaculum with caudally directed apophysis.

Differential diagnosis.—*Theristus* (*Theristus*) *rhynchonemoides* n. sp. is most closely related to *T.* (*T.*) *problematicus* (Allgen, 1928) but differs from it in the lack (or minuteness) of cephalic setae, smaller amphids, reduced size of cervical setae on narrow portion of neck, longer somatic setae and a shorter, less pointed tail.

Holotype.—Male on slide M40C.

Allotype.—Female on slide M40D.

Paratypes.—Three males on slide M40E.

Occurrence.—Collected May, 1958, by N. Sansing. Specimens washed from sand collected below low tide mark. Habitat exposed. Salinity 26.9$^0/_{00}$.

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THIN PREPARATIONS OF HEMOPOIETIC TISSUES[1]

VIBEKE E. ENGELBERT

Abstract

Previous papers dealing with behaviors and activities of blood cell nuclei have been recording mainly results with the imprint method and observations of blood cells cultured in vitro. The present paper demonstrates that the Feulgen "gentle squash" method as well as digestion with *N* HCl followed by toluidine blue staining at pH 4 reveals the same nuclear behaviors as the imprint method. Imprinting on millipore filters with subsequent fixation in Zenker's acetic solution followed by the Feulgen or toluidine blue staining method (the latter with or without previous *N* HCl digestion) is reported for the first time. The millipore imprint preserves a thicker layer of cells than imprinting on glass slides. Also cell stages which may not easily stick to glass seem to adhere well to the millipore filter. The filter imprint may in places present as many cell layers as a thin slice cut on the microtome, but without the disadvantage of having large or elongated cell stages cut into pieces by the microtome knife.

Introduction

The writer believes that the confusion in the literature regarding development of blood cells is largely due to the fact that many blood cell stages have been ignored or discarded as stages of degeneration; Engelbert (4, 5, 6) and McMillan and Engelbert (8, 9).

The blast cell nuclei can undergo great changes in shape during brief periods and pass from being a contracted more or less spherical structure to an elongated finally almost thread-like structure in a relatively short period of time (4, 5, 6, 7). These structural changes must be regarded as normal nuclear behaviors, which we must seek to understand in relation to the developmental history of blood cells.

The techniques reported in this paper reveal nuclear behaviors already reported earlier and lends further support to earlier statements about the dynamic aspect of behavior of blast cell nuclei.

A paper on blast cell nuclei observed in vivo and in vitro (9) is in preparation at present, so no further descriptions will be given here.

Materials and Methods

The present paper is only a section of recent experimental work which was undertaken in order to extend and expand earlier work. The earlier work dealt with the effect of foreign proteins on developmental stages of blood cells in the spleen, lymph nodes, and thymi of rabbits of farm stock. The present paper only reports the techniques used and the results obtained from the fixed stained preparations.

The animals were purchased as litter mates, usually six per litter at about 6 weeks of age. Four to five animals of each litter were injected, the rest were kept as controls without being submitted to any form of injection.

[1]Manuscript received August 18, 1960.
Contribution from Department of Zoology, University of Toronto, Toronto, Ontario.

The injections used were horse serum and horse serum ultrafiltrate. The horse serum was obtained either as neutral, sterile fresh serum provided through the courtesy of Dr. R. C. Parker, Department of Microbiology, or as vials of horse serum purchased from Difco Laboratories, Detroit, U.S.A. The horse serum ultrafiltrate was also obtained from Difco Laboratories, who describes the product as "a protein-free solution containing 0.001 per cent Phenol Red indicator". The protein-free injection provided a group of animals subjected to injection but not to foreign protein. Thus a second control group separate from the animals which did not receive any injection was provided.

The injections were given into marginal ear veins with a No. 26 needle, at the rate of 10 ml per 1800 g of animal. The rabbits weighed between 2000 and 3000 g when injected.

Forty-eight hours after the injection, the rabbit was killed. A blow on the back of the neck, causing fracture, was the method of killing used at first. However, this method often caused internal bleeding; furthermore, the shock caused the spleen to contract and to expel most of its fluid contents.

The above method of killing was therefore stopped and instead we resumed a practice we had used in earlier experiments, namely killing the animal with an intravenous injection of Ibatal (4). In this way the animal died easily and in a relaxed condition. Peristalsis and even a feeble heart beat were usually maintained until after spleen, lymph nodes, and thymus were removed in the animals killed with Ibatal.

The imprints were made on thoroughly cleaned slides by gently touching a freshly cut surface of the organ to the glass. Fixation was made in acetic alcohol 1:3 for 5 to 8 seconds followed by quick rinsings in two changes of absolute ethyl alcohol, then the slides were allowed to dry. Subsequently they were stained, either by the Feulgen method or using toluidine blue. The toluidine blue stain was used according to Morrison (10), who was the first to adopt and modify Bonhag's (1) techniques for use on imprints of blood cells. The imprints were stained in toluidine blue O (N.A) at 37° C for 30 to 40 minutes in a 0.05% stain solution in McIlvaine's citric acid – disodium phosphate buffer at 1/10 strength at pH 4.0 (see Pearse (11)).

Tissues to be treated with the Feulgen "gentle squash" method were fixed as very thin slices in acetic alcohol 1:3 for 15 minutes at room temperature or for 30 minutes or even overnight at 4° C. The pieces were then put into *N* HCl at 60° C for 15 minutes; then placed in Schiff's reagent at room temperature for 1 hour; then finally submitted to a careful teasing out on a clean slide in 45% aqueous acetic acid on the stage of a dissecting microscope.

When counterstaining was necessary, a 0.5% (or less) solution of fast green (FCF) in 45% acetic acid was used instead of 45% acetic acid alone as a medium for the operation of teasing the tissue apart. A cover slip was put over the teased preparation, which then was observed with the aid of a compound microscope. Then if deemed necessary a gentle pressure was exerted on the cover slip to press apart cells which had not separated. The cover slip was put on so one of its corners protruded outside the slide. The slide with cover slip on was now placed in tertiary butyl alcohol and left there until the cover slip fell off. Then both cover slip and slide was put into tertiary butyl alcohol and

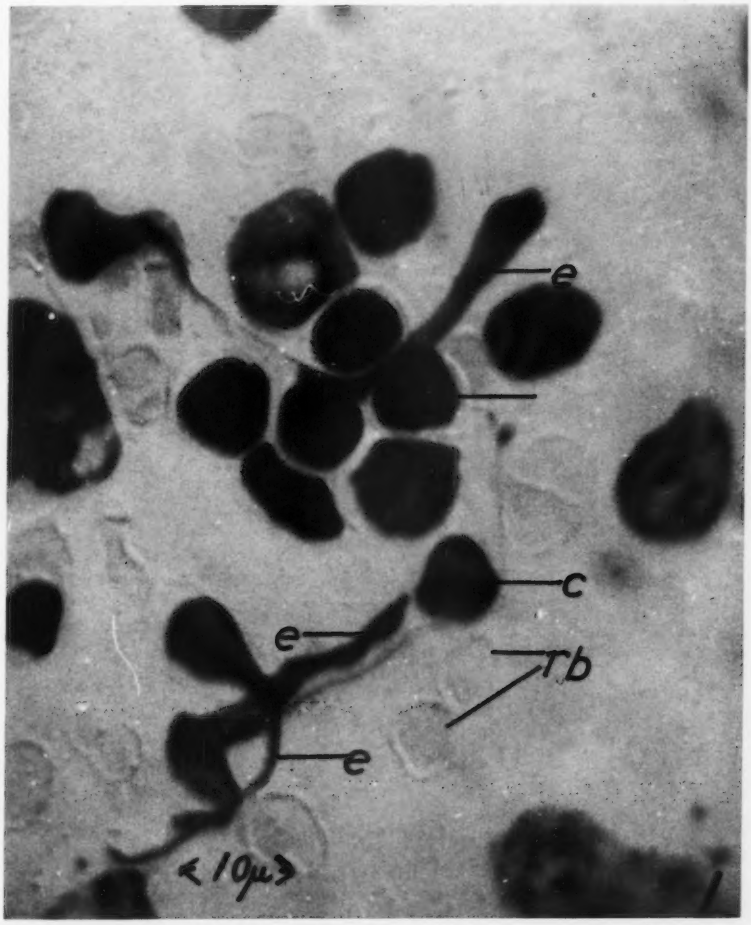


FIG. 1. Blast cell nuclei, extended (*e*) and contracted (*c*) stages. Red blood cells (*rb*), Imprint from spleen of rabbit killed 48 hours after injection of normal, sterile, fresh horse serum. Z. A. Dig. Tol. B.

Photomicrographs. Leitz ortholux microscope used for all figures. Objective 45×, ocular 10× used for Figs. 1, 2, and 3. Obj. 90×, Fig. 4. Leica camera with Micro Ibso attachment. Kodak High Contrast copy film used for all photographs.

Ac. A. Acetic alcohol 1: 3.
Z. A. Zenker's fixative with acetic acid.
Dig. Digested in *N* HCl.
Tol. B. Toluidine blue stain (N.A.).
Feulg. Feulgen nucleal stain.

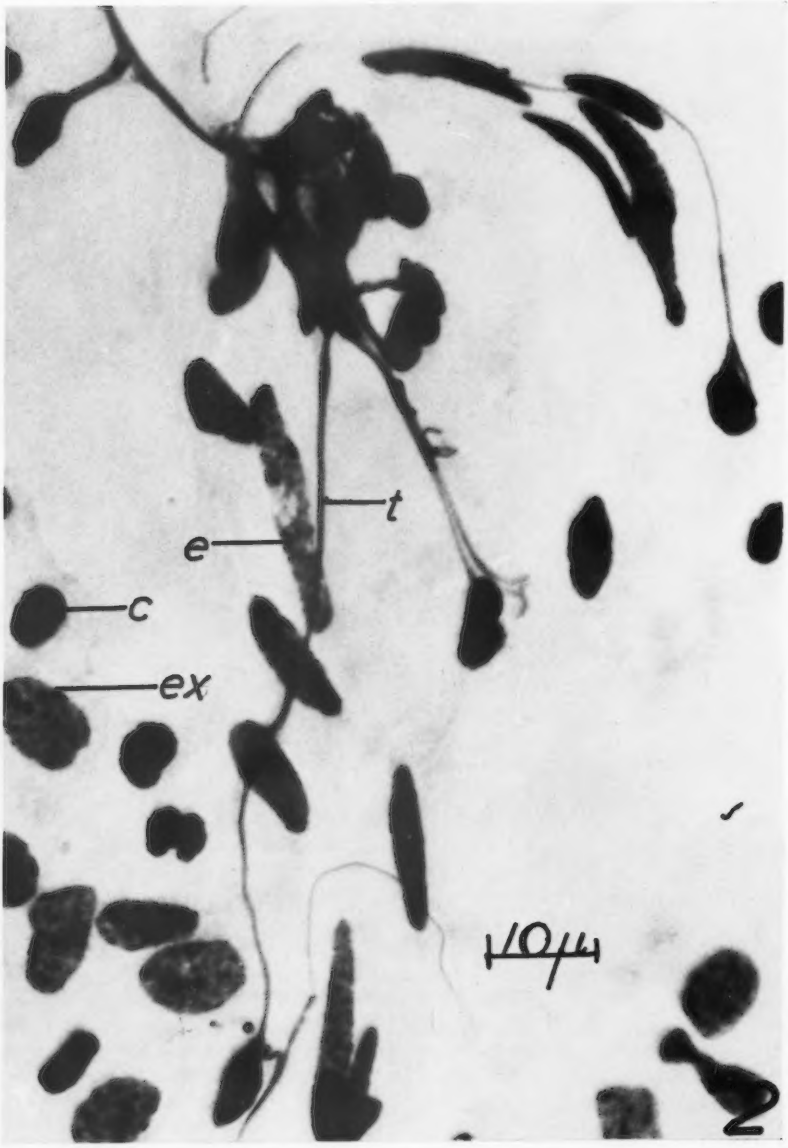


FIG. 2. Thread-like (*t*), elongated (*e*), contracted (*c*), and partially expanded (*ex*) blast cell nuclei. Spleen of female rabbit 48 hours after injection with normal, sterile, fresh horse serum. A. A. Feulg. "gentle squash" preparation.

xylol 1:1 for 5 minutes, then into pure xylol for 5 minutes, after which both slide and cover slip were mounted as usual.

The imprints on millipore filters were fixed by putting the filter with the imprint downwards into Zenker's acetic fixative. The millipore filters have a pore size of 0.45 μ and were obtained from British Drug House, Toronto.

After 5 minutes' fixation, the millipore imprints were washed in running water for 30 minutes, then rinsed in distilled water and stained in toluidine blue in the same manner as the imprints on slides. Malinol mounting medium from Chroma-Gesellschaft, Germany, purchased through Watzka Company, Montreal, was used to mount the millipore filters on a slide under a cover glass. Tertiary butyl alcohol as used by Bonhag (1) after the toluidine blue stain was found to work well also on the millipore filter imprints. Methyl alcohol destroys the filter (see catalogue on millipore filters); 95% and absolute ethyl alcohol can be used for brief periods. Millipore filter imprints fixed in Zenker's acetic solution were also stained with the Feulgen nucleal stain. From the rinse in distilled water after fixation, the filters were put into *N* HCl at 60° C for 10 minutes. They were then rinsed in distilled water and put into Schiff's reagent for 1 hour. Then followed three rinses of 3 minutes each in fresh SO_2 water and thereafter a 5 minutes' wash in running tap water; then a quick rinse in 95% ethyl alcohol, 2 minutes in absolute ethyl alcohol, 2 minutes in absolute alcohol and xylol 1:1; finally three changes in purexylol of 5 minutes each, followed by mounting with malinol. The millipore filters remain transparent and allow observation with high power objectives.

Discussion of the Methods Used

The imprint method and the Feulgen "gentle squash" method were used on the same material in order to see if the similarities of various morphological cell stages would be preserved with both methods. Although the imprint method has been used for many years by hundreds of workers it is sometimes being blamed (especially by people not using the method) for creating artifacts in cells and nuclei. This is especially the case when the cells and nuclei do not fit the classical image in the spectator's mind.

In our experience, after having observed many thousands of imprint preparations, the only artifact caused by cell damage is the squashing out of more or less delicate spherical cell stages such as the ones commonly called "basket cells". However, an experienced operator with a sufficiently "light hand" can prepare imprints without damaging the "basket" or similar delicate cell stages. The damage, if it occurs, is easily diagnosed in the fixed, stained imprints. With the smear method the "basket cells" and many other delicate stages are usually always damaged and have therefore been termed degenerating cells. This was pointed out by Cronkite (2) *et al.*, who found that "basket cells" far from being degenerating are actively synthesizing DNA and therefore cannot be "senescent degenerating cells". The "basket cell" is a cell stage that shows a rather loose, partly network-like nuclear pattern.

In this laboratory we believe "basket cell" stages such as the one shown in an earlier paper (4), Fig. 6, and identified with the label *pch* is a blast cell (it was called myeloblast by us in earlier papers). The loose network-like chro-

matin is easily pressed apart or damaged in smears or in imprints if they are not made with a sufficiently "light hand". Other blast cell stages that are equally delicate are shown in the same paper (4), Figs. 19 and 20. These particular stages are from imprints fixed in acetic alcohol 1:3, hydrolyzed, and stained according to DeLamater's (3) modified Feulgen method thus revealing nuclear structures composed of DNA.

The small delicate groups of DNA material (4, Figs. 19, 20) thus stained each represents what we, in this laboratory, call a "basic nuclear unit". It can be clearly seen that some of the basic nuclear units are undergoing mitotic divisions, the respective "chromosomes" being very small. Cells such as these are also very delicate and it is quite possible that such cells represent stages similar to the cells in Fig. 3B of the paper by Cronkite *et al.* (2), and shown by these workers to synthesize DNA.

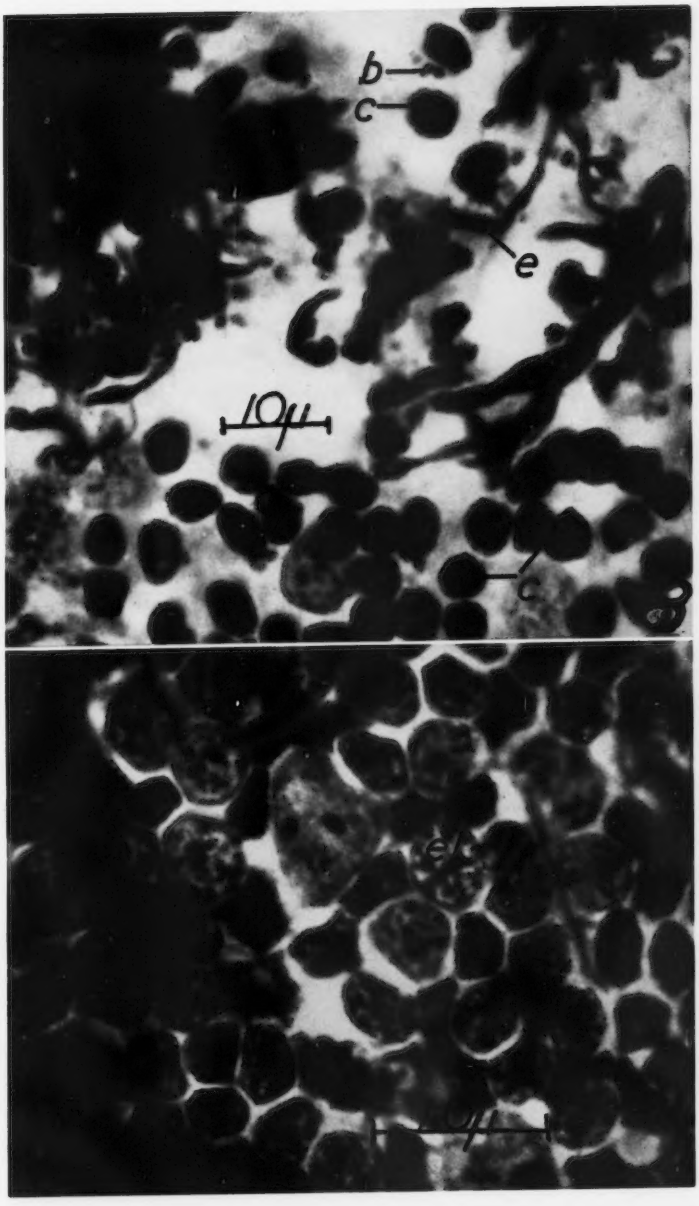
Another cell stage which never would be preserved in a smear or in a microtome section is the one published by the author in 1958 (5) and at that time illustrated in Figs. 4 and 5. In both these figures a long, delicate nuclear filament or tube is seen containing basic nuclear units *mi*. Only a carefully carried out imprint technique would preserve such a delicate and long (over 170 microns) structure. Harsh and careless techniques are in the author's opinion to be blamed for destroying much important morphological evidence involved in the developmental history of blood cells.

The Feulgen "squash method" is in most cases today not really carried out as a harsh "squash" method, where a great deal of pressure is exerted on the cellular elements. Rather, it is a method whereby thoroughly fixed small pieces of tissue are treated with the Feulgen nucleal stain and thereafter exposed to a gentle teasing apart with the aid of a good dissecting microscope. A very gentle pressure on the cover slip may follow to separate cell layers still adhering to each other. The method is used today by thousands of workers and for many different kinds of tissues. Certainly when small thin slices of hemopoietic tissue is first well fixed in acetic alcohol 1:3 or Zenker's acetic fixative and then submitted to the usual mild hydrolysis followed by immersion in Schiff's reagent before the teasing apart takes place, it is most unlikely that the fixed nuclei can become altered in appearance while the cells are being gently spread apart. Both the imprint method and the Feulgen "gentle squash" method, if handled properly by experienced workers, can reveal cellular and nuclear details which are not possible with sectioning techniques. These methods therefore are well worth testing for workers who have not tried them before.

Imprint technique on millipore filters has the advantage over imprints on glass slides that cell stages which do not adhere easily to glass adhere well to the filter. The millipore imprint is usually composed of more than one cell layer, so it represents, as it were, a thin section without the disadvantage of having cells cut by the microtome knife. Thin elongated stages of nuclei may

FIG. 3. Blast cell nuclei in various stages of elongation (*e*) and contraction (*c*). Free basic nuclear units (*b*). Mesenteric lymph node from male rabbit killed 48 hours after injection of horse serum ultrafiltrate. A. A. Feulg. "gentle squash" preparation.

FIG. 4. Imprint on millipore filter of thymus of 9-day-old male rabbit. Z. A. Tol. B. Note presence of many different cell stages. An elongated nucleus can be seen at (*el*).



Engelbert—Can. J. Zool.

not be so easily seen in between the layers of cells, but here and there they can be located, see Fig. 4 *el*. Spherical or rounded cell stages with more or less cytoplasm are beautifully preserved as seen in the same figure. The millipore imprint presents, as it were, a bridge to fill the gap between sections and the thinner preparations, such as imprints on slides and the Feulgen "gentle squash" preparations, where whole cells are needed.

One disadvantage, however, of the Feulgen "gentle squash" preparation is the ease with which the nuclei wash off after the squash is made and before the final mounting is accomplished. The imprint method where the cells are applied to the glass slide before they are fixed has therefore an advantage over the "gentle squash" method. The fact that the imprint on glass usually holds only one layer of cells is also an advantage.

Illustrations

Figure 1 is a photomicrograph of an imprint of rabbit spleen from a rabbit killed 48 hours after intravenous injection of fresh horse serum. The preparation was digested in *N* HCl at 37° C for 3 hours (see Morrison (10)).

It will be noted that nuclear morphology as already reported (4, 5, 6, 7, 8) range from elongated nuclei (*e*) almost ending in a pointed tip to contracted (*c*) darkly staining stages. Partially extended stages (*ex*) are also present.

That this nuclear morphology is not a product of the imprint method can be readily seen when Figs. 2 and 3 are observed. Both these two illustrations are from Feulgen "gentle squash" preparations from, respectively, spleen and mesenteric lymph node.

Elongated nuclei are present in the squash preparations as frequently as in the imprint preparations and stages of nuclear behavior can be followed in both types of preparations. A photomicrograph from an imprint of rabbit thymus on millipore filter stained with toluidine blue without previous digestion with *N* HCl is shown in Fig. 4.

It will be noted that nuclear and cytoplasmic details are well represented in this preparation, an elongated (*el*) nucleus can also be seen.

In conclusion, it should be pointed out that the techniques presented in this paper support the observations made earlier (4, 5, 6, 7, 8) using the imprint method on glass slides, then quickly fixing the wet imprints over iodine vapor, and finally staining with May-Grünwald plus Giemsa stain.

Acknowledgments

The National Research Council, Ottawa; the Ontario Research Council; and the University of Toronto are supporting these researches on blood cell development. Sincere gratitude is extended for these grants.

It is a pleasure to acknowledge the assistance and technical help provided by Dr. D. B. McMillan, Mrs. J. W. T. Dandy, and Miss Margaret Cooper.

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THREE TREMATODES FROM THE HARLEQUIN DUCK[1]

HILDA LEI CHING

Abstract

Three trematodes from the digestive tract of two harlequin ducks, *Histrionicus histrionicus pacificus*, were collected at Friday Harbour, Washington. They are *Paramonostomum histrionici*, n. sp. (Notocotylidae), and two microphallids, *Pseudospelotrema japonicum* Yamaguti, 1939 (new locality record) and *Spelotrema pygmaeum* (Levinsen, 1881) Jagerskiold, 1901 (new host and locality record).

Introduction

The following report is based on collections of intestinal trematodes from two harlequin ducks, *Histrionicus histrionicus pacificus*, shot and identified by Robert Dickerman at Friday Harbour, Washington, in 1959.

The worms were fixed in formalin – acetic acid – alcohol, stored in 70% alcohol and stained with Semichon's acetic carmine. Drawings were made with the aid of a camera lucida; measurements are in millimeters.

Family Microphallidae

1. Pseudospelotrema japonicum Yamaguti, 1939

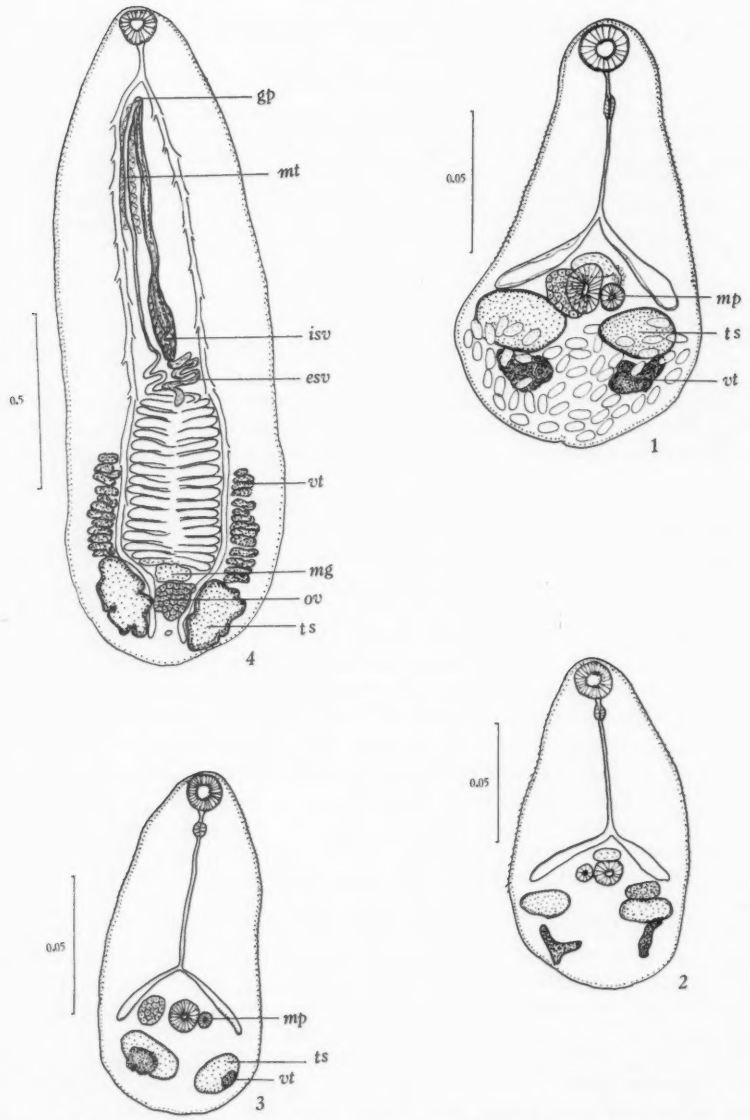
Nineteen specimens were collected from the two birds (2 in one, 17 in the other). Measurements of organs and body size were similar to those given by Yamaguti (3) but eggs of five specimens measured 18–27 by 10–13 μ instead of the 24–29 by 13–14 μ quoted. Large gland cells surround the muscular metraterm but were not mentioned previously. This is the first report of the worms in North America; they were described from the same host and from *Melanitta fusca steinegeri* in Japan and from *Tringa incana* in USSR.

2. Spelotrema pygmaeum (Levinsen, 1881) Jägerskiold, 1901

Eight very small worms were found in equal numbers in the two birds and resemble *S. pygmaeum* most in body shape, extent of ceca, ratio of male papilla to ventral sucker, and egg sizes (Fig. 1). The body shape varied from that shown in Fig. 1 to that in Fig. 2. The size range of these specimens is more nearly that of *S. claviforme* (Brandes, 1888) Nicoll, 1907. Measurements of six specimens, average in parentheses: length, 0.211–0.313 (0.273); width, 0.114–0.217 (0.156). Oral sucker, 0.027–0.039 (0.034); ventral sucker, 0.026–0.032 (0.028) in transverse diameters. Prepharynx lacking to 0.017 (0.008); esophagus, 0.065–0.088 (0.076) in length. Male papilla, 0.018–0.021 (0.019). Ovary, 0.026–0.045 (0.036); testes, 0.026–0.058 (0.050) in transverse diameters. Eggs, 20–25 by 9–12 μ (22 by 10) in length and width.

The trematodes appear to be cosmopolitan in distribution in a variety of birds. Belopolskaya (1) reported that sporocysts containing metacercariae of *S. pygmaeum* were found in *Littorina rudis* and *L. obtusata*. Two of over

[1]Manuscript received March 16, 1961.
Contribution from the Department of Zoology, University of British Columbia, Vancouver, British Columbia.



FIGS. 1–2. *Spelotrema pygmaeum* from *Histrionicus histrionicus pacificus*. Fig. 1. Ventral view of adult. Fig. 2. Dorsal view on smaller scale than Fig. 1.

FIG. 3. *Spelotrema* sp., metacercaria from *Littorina scutulata*.

FIG. 4. *Paramonostomum histrionici*, n. sp. from *Histrionicus histrionicus pacificus*.

ABBREVIATIONS: *esv*, external seminal vesicle; *gp*, genital pore; *isv*, internal seminal vesicle; *mg*, Mehlis' gland; *mp*, male papilla; *mt*, metraterm; *ov*, ovary; *ts*, testis; *vt*, vitellaria. Numbers on scales indicate millimeters.

five hundred *L. scutulata* examined at the Friday Harbour Laboratories by me were infected with yellow sporocysts containing *Spelotrema*-like metacercariae (Fig. 3). The metacercariae were slightly smaller than the smallest adults found in the ducks. Additional infections of *L. scutulata* and *Spelotrema* sp. were found in Vancouver, B. C. The sporocysts were fed to two mice; immature worms were recovered after 24 hours and ovigerous forms, after 72 hours. The mature worms had few eggs in the uteri and were larger than those found in the harlequin duck. Their size range, however, is still within that given for *S. pygmaeum*.

Family Notocotylidae

3. Paramonostomum histrionici n. sp.

Host.—*Histrionicus histrionicus pacificus.*

Location.—Intestine.

Locality.—Friday Harbour, Washington.

Frequency.—Eighteen mature specimens, the majority in one of two birds examined.

Holotype.—U.S. National Museum No. 59547. See Fig. 4 of holotype.

Description (based on five specimens, average in parentheses).—Body elongate, edges curled ventrally in life, posterior end rounded, length, 1.357–1.938 (1.609); width, 0.433–0.609 (0.552). Body surface finely spined, lacks ventral glands. Muscle fibers well developed throughout body. Oral sucker, 0.108–0.120 (0.115) in transverse diameter. Pharynx lacking; esophagus, 0.063–0.085 (0.077) in length. Intestine bifurcates 9–13% from the anterior edge of the body. Intestinal ceca with short lateral branches, extend posteriorly between testes and ovary and end at the posterior edge of the ovary. Genital pore slightly posterior to intestinal bifurcation. Testes elongate, lobed to a moderate degree, symmetrically placed in the posterior part of the body, 0.165–0.228 (0.203) long and 0.114–0.182 (0.132) wide. External seminal vesicle contorted, joins the cirrus sac which contains the short, coiled, internal seminal vesicle, club-shaped prostatic vesicle, prostatic cells, ejaculatory duct, and unarmed cirrus. Cirrus sac elongate and club shaped, 0.427–0.644 (0.565) in length, reaching to mid-body. Ovary irregularly lobed and somewhat round, 0.085–0.114 (0.070) in diameter. It is located between the testes at their anterior level. Mehlis' gland complex about one-half the size of the ovary, proximal loops of the uterus form a seminal receptacle. Coils of uterus number 16–18; the metraterm is surrounded by numerous gland cells and is one-half the length of the cirrus sac, 0.228–0.343 (0.270). Vitellaria are transversely oval follicles that extend 0.313–0.427 (0.357) from the ovary. They lie in the posterior 30–40% of the body and are extracecal. Eggs, exclusive of polar filaments, measure 17–22 by 9–13 microns. Polar filaments in one egg measured over 100 μ. Excretory pore dorsal, midway between posterior edge of the body and ovary, in one specimen surrounded by about nine cuticular spines.

Comparisons.—Of the reported species of *Paramonostomum* with elongate bodies, *P. histrionici* resembles *P. malerischi* Dunagan, 1957; *P. actitidis* Cable, Connor, and Balling, 1960; *P. elongatum* Yamaguti, 1934; *P. echinum* Harrah, 1922; and *P. bucephalae* Yamaguti, 1935. *P. histrionici* differs from *P. malerischi*, a species reported from the emperor goose in Alaska, in the

location of the genital pore and the shorter extent of the vitellaria. This species differs from *P. actitidis* and *P. elongatum* in body size and the length of the metraterm in relation to the length of the cirrus sac. Moreover, *P. actitidis* has numerous prostate cells in the basal portion of the cirrus sac and 10 to 12 uterine coils. *P. elongatum* is not spined and has smooth ceca. *P. histrionici* differs from *P. echinum* in the size of the oral sucker in relation to body size, location of the cirrus sac in the anterior half of the body, and the number of uterine folds (16–18 in contrast to about 22 in Harrah's figure of *P. echinum*). From *P. bucephalae*, *P. histrionici* differs in the lack of tubercles on the cirrus and pars prostatica, and in the shorter extent of vitellaria. *P. echinum*, *P. bucephalae*, and *P. histrionici* all have metraterm lengths one-half the length of the cirrus sac. Smith (2), in his study of *Quinqueserialis hassalli* (McIntosh and McIntosh, 1934) Harwood, 1939, showed that the metraterm length could be one-half or as long as the cirrus sac length. The number of uterine loops also varied from 10 to 15. In the author's opinion, the metraterm length relative to the cirrus sac length and the number of uterine loops may be used for differentiation until age, host, and morphological variations of this genus can be studied.

Acknowledgments

The author expresses appreciation to Robert Dickerman for collections of birds, to Dr. Robert Fernald of Friday Harbour Laboratories for the use of facilities, and to Dr. J. R. Adams for research space and equipment.

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NOTES

FURTHER OBSERVATIONS ON DIPLOTRIAENA IN BIRDS*

ROY C. ANDERSON

Since publication of a revision of *Diplotriaena* Henry and Ozoux, 1909 (Filarioidea, Diplotriaenidae) (1) additional specimens of the genus have been collected from birds in Ontario. Some of these records are worth reporting as they add to knowledge of the host and geographic distribution of several species of *Diplotriaena*.

D. ozouxi Henry and Ozoux, 1909, previously reported once from *Vermivora ruficapilla* (Wilson) (Parulidae), has now been found in another bird of this species (Ontario Research Foundation Nematode Collection No. 359). Specimens from two *Quiscalus quiscula versicolor* Vieillot (Icteridae) (Nos. 137,138) are referred to *D. bargusinica* Skrjabin, 1917, although the oesophagus is of the undivided type. A number of molting fourth-stage *Diplotriaena* (No. 121), probably belonging to *D. bargusinica*, were found in the heart and aorta of a *Hylocichla ustulata swainsoni* (Tschudi) (Turdidae) estimated to be about three weeks old but they may have migrated there from the lungs after death of the host. A female from *Riparia riparia* (L.) (Hurundinidae) (No. 132) has been assigned to *D. obtusa* (Rudolphi, 1802).

New host records are as follows.

(a) For *D. agelaius* (Walton, 1927). Icteridae: *Quiscalus quiscula versicolor* Vieillot (No. 116); Parulidae: *Wilsonia canadensis* (L.) (Nos. 120, 360, 362), *Dendroica magnolia* (Wilson) (No. 361), *Dendroica pensylvanica* (L.) (No. 131), *Vermivora ruficapilla* (Wilson) (No. 130). This species was previously reported in *Agelaius phoeniceus* and *Ostinops decumanus* of the Icteridae and *Seiurus aurocapillus* of the Parulidae. Specimens from the latter species were previously named *D. translucidus* (Anderson, 1956). Study of additional specimens has not necessitated revision of the view that *translucidus* is a synonym of *agelaius*.

(b) D. tricuspis (Fedtschenko, 1874). Corvidae: *Cyanocitta cristata* (L.) (No. 363). There remains no authentic record of *tricuspis* in other than Corvidae.

(c) D. bargusinica Skrjabin, 1917. Turdidae: *Hylocichla fuscescens* (Stephens) immature (No. 367); unlike specimens from *Quiscalus*, these have the oesophagus of the divided type.

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RECEIVED FEBRUARY 27, 1961.
DEPARTMENT OF PARASITOLOGY,
ONTARIO RESEARCH FOUNDATION,
TORONTO 5, ONTARIO.

*This work was made possible by a research grant to the Ontario Research Foundation by the Department of Commerce and Development of the Province of Ontario.

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